OBITUARY NOTICE.

HORACE TABBERER BROWN.

BORN *July 20th, 1848.* DIED *February 6th, 1925.*

H. T. BROWN was the posthumous son of Benjamin Tabberer and he assumed the surname of his stepfather, Edwin Brown of Burton-on-Trent, a banker, and in his leisure hours a naturalist of considerable attainments, who, by his sympathy and encouragement, exerted a great influence on the life of his step-son. At the early age of 12 Brown became interested in science. At the time he was receiving a grammar school education he realised that in chemistry he had found his *métier* and he devoted most of his time to acquiring knowledge in this direction. His interest in chemistry was fostered by Böttinger and Griess, who were then in Burton. In 1865 Brown became a student at the Royal College of Chemistry at Oxford Street, Hofmann still being professor there. One of the junior assistants at the College was Cornelius O'Sullivan and a fellow-student of Brown's was his lifelong friend Dr H. E. Armstrong.

After two terms at the College of Chemistry Brown entered in 1866 the brewery of Worthington & Co. His first scientific work was concerned with analyses of the Burton waters and the treatment of crude sewage. At the age of 25 he became manager of the whole of the manufacturing department, a post he occupied until 1889 when he was appointed a managing-director. As there was but little time to spare for laboratory work Brown selected John Heron as his assistant. The first fruits of this combination was a re-examination of the whole question of starch and its transformation. This work on starch was continued for many years in collaboration successively with J. Heron, G. H. Morris, J. H. Millar and others. In all some nineteen papers were published on this fascinating and intricate subject.

In 1883 Brown took up the study of geology, being led to it by some pressing requirements in connection with the water supply of Burton. The result of this work, which entailed a good deal of field surveying, was embodied in a paper on the Permian Rocks of the Leicestershire Coalfield. He appeared to have felt that too much of his time was being spent over geology in view of the many other problems which suggested themselves to him. The malting process in those days was completely empirical and Brown turned his exclusive attention to the physiology and anatomy of germinating seeds and their bearing on industrial malting.

In 1890 he and Morris published in the *Journal of the Chemical Society* their classical work on "Researches on the Germination of the *Gramineae*,"

a paper which he regarded as his most important and original effort. The results of this work were far reaching and it affords a good example of the manner in which a careful study of an industrial process can react on pure science. In 1893 Brown found that hop strobiles contained a diastatic enzyme and this fact was the starting point of his work connected with the chemistry and physics of assimilation in green plants. In 1893, with Morris, a paper entitled "A Contribution to the Chemistry and Physiology of Foliage Leaves" was read before the Chemical Society, and this was the last work carried out at Burton.

Not long after his arrival in London Brown engaged J. H. Millar as his chemical assistant and the work on starch and its products of transformation was continued, the results being published in a well-known series of papers in the *Journal of the Chemical Society*. Brown also at this time, in conjunction with the late Spencer Pickering, investigated the thermo-chemistry of carbohydrate hydrolysis, which afforded interesting results. With F. Escombe he studied at the Jodrell Laboratory, Kew, some of the outstanding problems connected with the germination of barley. The work on the assimilation of atmospheric CO_2 by green leaves was continued and this led to a study of the phenomena of the diffusion of gases and liquids. The work was of great, if not of fundamental, importance, since it showed how it was possible to account for the sufficiently rapid ingress of atmospheric CO_2 necessary for the formation of carbohydrate on the assumption that the interchange of gases between the leaf and the outer air took place only through the stomatic openings and not through the whole area of the epidermis, a conclusion also reached by F. Blackman by the aid of a series of very beautiful experiments. They also attempted to determine the mode of interchange of energy between the leaf and its environment and an endeavour was made to ascertain what proportion of the solar radiant energy is actually utilised by the chloroplasts in carbohydrate production within the leaf. The results were embodied in the Bakerian Lecture for 1905.

In 1901 Brown established the Guinness Research Laboratory in Dublin for the purpose of studying some of the more important problems connected with the brewing business of that firm. Much of the purely scientific portion of the work was printed for private circulation. Between the years 1907 and 1913 three papers were published in the *Journal of the Institute of Brewing*, on the "Nitrogen Question in Brewing," some of which had important technical applications.

It is significant of Brown's remarkable energy and spirit of enquiry that during occasional visits extending over a number of years to a fruit and wine farm he acquired for one of his sons in Cape Colony, he introduced modern methods for increasing the yield of deciduous fruits by artificial cross-fertilisation of the trees. He also succeeded in improving the methods of wine-making at the Cape and published an account of his investigations on this subject in the *Journal of the Institute of Brewing* (1914).

In 1908 he was appointed a member of the Royal Commission on Whisky and Potable Spirits, and in 1909, in conjunction with the late Sir Edward Thorpe, he investigated the method of determining the original gravity in beers. This enquiry involved a great amount of work in a number of breweries in different parts of the United Kingdom and the final results were published in a series of reports. Corrected tables for the determination of original gravity were constructed and their use became official. In 1918 Brown read his last paper to the Chemical Society on his work on Diffusion.

From the years 1870 to 1918 Brown published 90 papers and memoirs, not a few of them being regarded as classics. The value of his work was recognised and his election as a Fellow of the Royal Society in 1889 was the forerunner of a number of subsequent distinctions. He was awarded the Longstaff Medal of the Chemical Society in 1894, a Royal Medal of the Royal Society in 1903 and the Copley Medal in 1920. For a time he was Treasurer and afterwards Foreign Secretary of the Chemical Society, and only failing health prevented him from accepting the Presidentship of that Society.

JULIAN L. BAKER.

XXIII. THE DETERMINATION OF GUANIDINES IN URINE AS PICRATES

By JOHN SMITH SHARPE.

From the Institute of Physiology, Glasgow University.

(*Received December 4th, 1924.*)

Burns and Sharpe [1917] described a marked increase in the amount of guanidine and methyl-guanidine in the blood and urine of dogs in tetania parathyreopriva and in the urine in idiopathic tetany in children.

The method then used was based upon that of Koch and involved the use of mercuric chloride. It has been criticised by Greenwald [1924] on the ground that by the use of the mercuric salt creatine may be split into guanidine. Further, the precipitation with auric chloride is unsatisfactory as the salt is easily decomposed even by light; and, as the gold salt of guanidine is not very insoluble, there is a considerable loss. The objection that creatinine in the urine may in our investigations have been estimated along with the guanidine seems to be negatived by the finding of Burns [1917] that after administration of guanidine there is little if any increase of creatinine.

Later Findlay and Sharpe [1920], and Nattrass and Sharpe [1921] recorded the presence of methyl- or dimethyl-guanidine in the urine in two cases of adult tetany. The method used depends upon precipitation by tannic acid and subsequent precipitation of the bases with picric acid. By this method the objections to the process previously used are eliminated. The danger that creatinine, potassium and ammonium picrates may be precipitated along with guanidine picrate has to be considered, but the difference in solubility and character of the crystals obviates this danger.

This method has also been criticised by Greenwald on the ground that the precipitate may have in part consisted of creatinine and ammonium picrate [1924, p. 332].

Since the publication of the method in 1920, improvement in some particulars has been the object of study, and the modification now described has been devised.

Method.

250 or 500 cc. of urine were treated with a 10 % solution of tannic acid. This was added in small quantities at a time and shaken after each addition. The precipitant was added until the supernatant liquid appeared green in colour. The precipitate was allowed to settle and filtered off by suction. To the filtrate $Ba(OH)_2$ was added in excess, the precipitate of barium tannate

allowed to settle and filtered. To remove the excess of hydroxide a few cc. of a 1 % H_2SO_4 solution were added in slight excess; this excess was then neutralised by adding a cream of $BaCO_3$, testing with litmus. The filtrate from this was evaporated to a syrup which usually crystallised on cooling. When cool the mass was extracted thoroughly with absolute alcohol and filtered: about 60 cc. of alcohol were necessary for 250 cc. of the original urine. The alcohol was evaporated off and the mass again extracted with about 20 cc. of alcohol. All the guanidine bases, creatinine, traces of potash, soda and ammonia are in the extract. The alcohol was again evaporated off and the residue dissolved in 10 cc. water. A saturated aqueous solution of picric acid was now added until the total volume was about 20 cc. This was set aside to crystallise.

The following tests show the applicability of the method:

1. *Recrystallisation of mixed picrates.* 50 mg. creatinine hydrochloride and 50 mg. guanidine hydrochloride were dissolved in a few cc. of water and saturated picric acid was added to precipitate the bases. The precipitate was filtered and dissolved in about 20 cc. of water at about 80° and set aside to crystallise.

Recovery: 47·8 mg. (95·6 %) guanidine hydrochloride.

The creatinine picrate separated on further evaporation. Its presence, along with the guanidine or methyl-guanidine picrates may be recognised by Jaffe's test and its amount estimated by Folin's method.

2. *Recovery of guanidine from mixtures of urinary chlorides.* 50 mg. creatinine hydrochloride, 50 mg. guanidine hydrochloride, 100 mg. potassium chloride and 100 mg. sodium chloride were dissolved in a little water and the mixture was evaporated to dryness, as in the final stage of the process of extraction of bases. The crystalline residue was treated in the usual way with 15–20 cc. absolute alcohol, filtered, and the alcohol evaporated off. Guanidine and creatinine were retained in the filtrate along with a little potassium.

After evaporation 10 cc. of water were added, then 10 cc. of saturated picric acid solution. The guanidine was precipitated immediately. After the guanidine picrate had been separated by filtration further evaporation was allowed to occur and creatinine picrate was obtained. On still further evaporation to 10 cc. characteristic hexagonal needles of potassium picrate were obtained, but this was not estimated. A little more creatinine was obtained in this fraction.

Recovery: 43·8 mg. (87 %) guanidine hydrochloride and 27 mg. (43·4 %) creatinine hydrochloride.

Sodium picrate is so very soluble that it would be impossible to precipitate it in this way.

3. *Addition of guanidine and creatinine to normal urine.* 50 mg. creatinine hydrochloride and 50 mg. guanidine hydrochloride were dissolved in 250 cc. normal urine and the full process of extraction carried out. The creatinine was not estimated.

Recovery (in two similar experiments): 42·9 and 44·8 mg. (85·5 and 89·6 %) guanidine hydrochloride.

Should creatinine picrate appear along with the guanidine picrate it was found that a safe and reliable method of eliminating it was fractional crystallisation. The mixed picrates were heated with just sufficient water at about 80° to dissolve the less soluble guanidine salt; this was set aside and the guanidine picrate separated free from creatinine and potassium.

As an alternative to this method of crystallisation, the creatinine can be removed by adding pure blood charcoal in slightly acid solution. Folin and Denis [1916] claim that upwards of 90 % of the creatinine is thus eliminated.

Ammonium picrate is more soluble than the potassium salt and it comes down only in concentrated solutions. It is very easily identified by the deep colour of the crystals and by their very constant crystalline form.

Summary.

1. A method for the estimation of guanidine and methyl-guanidine is described. The process is simple and rapid and the proportion of guanidine recovered is nearly 90 %, whilst the separation from creatinine and potassium is complete. The photographs (Plate I) should assist in the identification of the picrates found in the various fractions of the final stage.

2. As mercuric chloride is not used the possibility of conversion of other bases (creatine) to guanidine is eliminated.

My thanks are due to Professor Noël Paton for advice and help in carrying out this work. The expenses were defrayed by a grant from the Medical Research Council.

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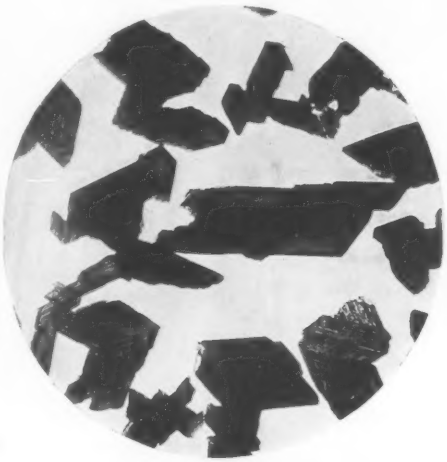
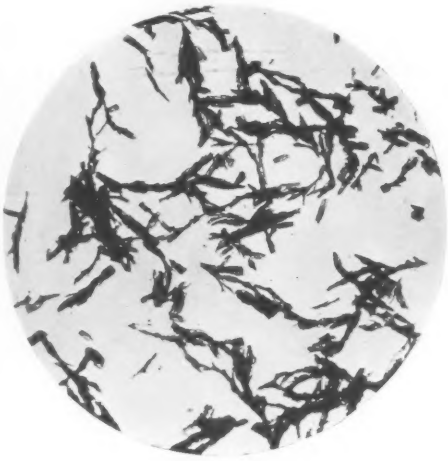


Guanidine picrate. × 150.

Methyl-guanidine picrate. × 150.



Dimethyl-guanidine picrate. × 150.

Creatinine picrate. × 150.

XXIV. THE DIAZO REACTION IN URAEMIC SERA.

By LESLIE FRANK HEWITT.

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(*Received January 12th, 1925.*)

The general concensus of opinion is that uraemia is due to the inability of the kidney to deal with certain normal or abnormal products of metabolism. Foster [1923] emphasises the selectivity of this retention by the kidney as contrasted with general retention of metabolic products (urinaemia). Thus, whilst in normal blood the residual nitrogen comprises some 10–20 % of the non-protein nitrogen present, in uraemic blood this value reaches 30–50 %. Attempts to identify the substance or substances responsible for the symptoms of uraemia have, so far, proved unsuccessful. Foster [1915] claims to have obtained from uraemic blood a material which caused the death of guinea-pigs; full experimental details have, however, not been published, and the amount of the material obtained and its properties are not given.

The diazo reaction may prove a valuable means of identifying at least one of the contributory factors in uraemia. Ehrlich [1882] and Pauly [1904, 1905] have described the red colour produced when a diazotised solution of sulphanilic acid is added to certain urines and the mixture rendered alkaline. Van den Bergh [1918] applied the diazo reaction to the detection and estimation of bilirubin in serum. When a dilute hydrochloric acid solution of diazotised sulphanilic acid is added to serum containing bilirubin a pink colour, turning green on addition of alkali, is obtained (*direct* reaction). In certain cases the reaction is obtained only when an alcoholic extract of serum is used (*indirect* reaction). This indirect reaction is conveniently conducted [McNee, 1923] as follows: 2 cc. of serum are poured into 4 cc. of 96 % alcohol, the coagulated proteins are filtered off and to 2 cc. of the clear filtrate are added 1 cc. of 96 % alcohol and $\frac{1}{2}$ cc. of dilute freshly-prepared *p*-sulphophenyldiazonium chloride.

Andrewes [1924], on applying this test to uraemic sera, observed the formation of a buff colour in place of the usual pink. This buff colour deepened gradually when allowed to stand for twenty-four hours, and when made alkaline with caustic soda this deepened buff colour changed to a bright rose red, which faded rapidly. Andrewes obtained this reaction with the sera from eight patients suffering from severe uraemia, but failed to obtain it with the

serum of patients suffering from other diseases. This has been confirmed by the present author; the reaction has been obtained in the case of twelve uraemic patients[1] with blood urea concentrations of from 240 to 692 mg. per 100 cc. (mean value 366 mg.). As found by other workers [Van den Bergh, 1918; Andrewes, 1924; Feigl and Querner, 1919] these nephritic patients showed very marked hypo-bilirubinaemia[2]. In view of the possible connection between the substance causing the diazo reaction and that causing the symptoms of uraemia it was decided to investigate the matter further.

The conditions affecting the reaction itself were first studied. The conclusions reached were as follows:

(i) The characteristic of the reaction is the evanescent pink colour obtained on adding caustic soda, since the buff colour is more difficult to detect in pigmented sera and may be obtained with blood other than uraemic.

(ii) In place of the tedious waiting for twenty-four hours for the development of the buff colour, the test may be made without delay by boiling the reaction mixture for 30 seconds before the addition of caustic soda.

(iii) Strong bases (*e.g.* potassium hydroxide and baryta) produce the pink colour when added to the buff solution, whereas weaker bases (*e.g.* sodium carbonate and ammonia) do not.

(iv) The buff solution has been kept for as long as two months without apparent decomposition, whereas the pink coloration may fade in a few seconds, or at most lasts an hour, according to the intensity of the colour. When the pink colour has once faded the buff colour cannot be recovered by addition of acid, whereas the pink solution becomes buff on acidification and pink again on being rendered alkaline. Thus the fading of the pink colour indicates a reaction not reversed by acids.

(v) Sera do not themselves give the reaction, an alcoholic extract must be used.

(vi) Oxalated plasma may be used in place of serum and is often more convenient. The cerebro-spinal fluid, however, appears not to give the test. In one case of severe uraemia the urea in the C.S.F. reached the concentration of 692 mg. per 100 cc., but the test was negative.

(vii) The intensity of the reaction does not follow the variations in the concentration of urea in the blood, although in general the intensity of the reaction roughly follows the severity of the uraemia.

(viii) The presence of acetic acid interferes with the reaction and may even inhibit the appearance of the pink colour entirely.

[1] The author is indebted to the following gentlemen for samples of uraemic blood: Dr G. A. Harrison, Dr R. D. Lawrence, Prof. Fraser, Dr Marrack and Dr Linder.

[2] Two infants of 4 and 6 months respectively with uraemia gave a negative test. This may possibly be due to a lower resistance than that of older patients, death therefore occurring at an earlier stage of the disease, before sufficient accumulation in the blood of the substance causing the reaction. It is noteworthy that these did *not* show hypo-bilirubinaemia.

Experiments on the substance causing the reaction.

(i) 24 cc. of an alcoholic extract of uraemic plasma (1 part of plasma to 2 of alcohol) were evaporated to dryness in an open dish; the residue was extracted with 2 cc. of absolute alcohol. Two drops of this extract were diluted to 1 cc. with a mixture of alcohol (2 parts) and water (1 part). This solution gave a strongly positive reaction with a diazotised solution of sulphanilic acid.

(ii) The substance is not removed from plasma or serum, when neutral, acidified or made alkaline, by ether.

(iii) 10 cc. of the alcoholic extract (66 % alcohol) were evaporated to one-third to remove the alcohol, made alkaline by the addition of a few drops of 40 % NaOH and extracted with 2 cc. of pure amyl alcohol. The aqueous layer showed a much diminished colour reaction, whilst the amyl alcohol gave a marked reaction. About six extractions of a deproteinised serum or plasma, made alkaline with either sodium hydroxide or sodium carbonate, with one-third the volume of amyl alcohol were sufficient to remove completely the substance causing the reaction. The amyl alcohol from such an extraction was removed by steam distillation, the material remaining in the aqueous residue.

(iv) The substance is entirely removed from an alcoholic extract by warming with a small quantity of absorbent vegetable charcoal and cannot be removed from the charcoal by warming with dilute acids or alkalis nor by extracting for ten hours with alcohol in a Soxhlet apparatus.

Possible identity of the substance causing the reaction.

The substance possesses basic properties since it is extracted by amyl alcohol from alkaline solutions only. It is soluble in water, dilute acids and alkalis, in ethyl and amyl alcohols in the dilutions employed, but it is not soluble in ether. It is not volatile in steam and is stable to moderate heating. It is irreversibly adsorbed by charcoal. It cannot be detected in the blood of normal persons nor in that of patients suffering from diseases other than uraemia.

The simpler aliphatic amines are excluded on account of their volatility in steam and solubility in ether. Solutions of urea, creatinine and trimethylamine fail to give the reaction. It is probable that cyclic amines alone give this diazo reaction. In support of the hypothesis that the substance in uraemic blood causing the reaction is a toxic base containing the benzene nucleus or the iminazole grouping, *e.g.* tyramine or histamine, the following evidence can be adduced:

(1) Amines containing the phenyl or iminazole group give an evanescent pink coloration with an alkaline solution of diazotised sulphanilic acid [Koessler and Hanke, 1919, 1922].

(2) They are not volatile in steam [Barger, 1914].

(3) They are stable to moderate heating.

(4) They are not soluble in ether, but are extracted by amyl alcohol from alkaline solution [Koessler and Hanke, 1924, 1].

(5) Tyramine increases the blood pressure [Dale and Dixon, 1909], a

common symptom of uraemia; and tyramine produces renal disease and experimental nephritis in rabbits [Harvey, 1911].

(6) Koessler and Hanke [1924, 2] showed that over 60 % of the faeces they examined, both normal and pathological, contained bacteria which decarboxylated tyrosine and histidine to yield the corresponding toxic amines. Since these two amino acids are common products of digestion the presence in the body of the amines derived from them is readily explicable.

(7) Bain [1909, 1910] claims to have isolated tyramine from normal and pathological urines. Alcoholic extracts of urines frequently give (with the diazo reagent) a very similar reaction to that of uraemic blood.

(8) It has been shown [Koessler and Hanke, 1924, 3] that the liver plays a very subsidiary part in the detoxication of these amines, thus leaving the kidney to deal with them; so that, in case of kidney damage, such amines will not be readily eliminated from the body.

It would thus seem that the substance causing the diazo reaction in uraemic sera and plasma possesses many of the properties of the cyclic amines; further these amines are normally present in the intestine, and, as their elimination from the body depends on the kidney, nephritis would be expected to interfere seriously with their elimination. The accumulation of these amines in the blood in uraemia would thus be anticipated and may well account for the diazo reaction given by uraemic blood and for certain of the symptoms of uraemia.

Summary.

(1) Andrewes' findings with regard to the diazo reaction in uraemic blood have been confirmed. It is possible to complete the reaction in one minute instead of twenty-four hours, by means of a slight modification.

(2) It is tentatively suggested that a cyclic amine, such as histamine or tyramine, may occur in uraemic blood and may cause the reaction.

The author wishes to express his deep indebtedness to Dr G. A. Harrison of the Hospital for Sick Children, Great Ormond Street, for providing the facilities whereby this work was carried out, and for his help and advice.

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XXV. STUDIES ON XANTHINE OXIDASE.

IV. RELATION OF XANTHINE OXIDASE AND SIMILAR OXIDISING SYSTEMS TO BACH'S OXYGENASE.

By SYLVA THURLOW.

From the Biochemical Laboratory, Cambridge University.

(*Received January 16th, 1925.*)

In two recently published papers, one by Haas and Hill [1923], the other by Haas and Lee [1924], the authors state the existence in milk not only of a substance which can reduce nitrates but of another substance which, in the presence of aldehyde and molecular oxygen, can oxidise nitrites to nitrates. They call this latter substance itate, and explain its activity in the following way. In the presence of itate the aldehyde reacts with atmospheric oxygen to form an aldehyde peroxide, the oxygen of which is activated by the peroxidase of milk and oxidises the nitrite. Since the nitrate reducing power of milk has been shown by Dixon and Thurlow [1924] to be due, not to a special substance with that characteristic property, but to the xanthine oxidase plus any one of its substrates, the question arose as to whether the nitrite oxidising power of milk might not be ascribed to the same system. A possible mechanism which would bring about the oxidation of nitrites in such a system would be a coupled reaction. The substrate of the xanthine oxidase, having been activated by the enzyme, is oxidised by molecular oxygen. During the course of this reaction active oxygen would be produced and might oxidise the nitrite. If this were true, then not only aldehyde but hypoxanthine and xanthine in the presence of milk and molecular oxygen should bring about the oxidation of nitrites. The following typical experiment showed that such was indeed the case.

In each of four tubes were placed 10 cc. of milk and 0·2 mg. $NaNO_2$. To the first was added 1 mg. of hypoxanthine, to the second 1 mg. of xanthine, to the third 0·5 cc. of 10 % acetaldehyde. To the fourth no substrate was added. The tubes were aerated at room temperature for 45 minutes and then tested for nitrite by the addition of Griess-Ilosvay reagent, which gives a pink colour in the presence of nitrite. The intensity of this colour is proportional to the amount of nitrite present, and is indicated in the table by a varying number of + signs. A negative test therefore indicates that all the nitrite has been oxidised. The results are given in Table I.

If then, as this experiment indicates, the oxidation of nitrite is being in some way brought about by the ordinary oxidase of milk the reaction should

occur upon the addition of any substrate of the enzyme, but not upon the addition of a substance not activated by the enzyme. The reaction should be inhibited by the same substances which Dixon and Thurlow have shown to inhibit the activity of the xanthine oxidase. Table I shows that in the presence of hypoxanthine, xanthine, aldehyde and adenine, all of which are activated by the oxidase of milk, nitrites are oxidised. The amount of nitrite oxidised is roughly proportional to the velocity with which the substrates are oxidised by the enzyme, as is shown in the table by comparison of the reduction-time of methylene blue by the various substrates. In the presence of those substances which are not activated by the oxidase, *e.g.* uric acid, guanine and caffeine, nitrites are not oxidised. Table I also shows that the oxidation of nitrites is inhibited by the same substances as inhibit the oxidation of hypoxanthine.

The effect of varying concentration of substrate was then investigated. Increasing the hypoxanthine concentration gave the inhibition expected from the results given in the previous papers, whereas increasing the aldehyde concentration increased the velocity of oxidation of nitrite, which result was also obtained in the previous work. An experiment typical of many is given in Table I. These results left little doubt that xanthine oxidase was a constituent of the system which was responsible for the oxidation of nitrites.

Table I.

Each tube in the aeration experiments contained 10 cc. milk, 0·05 mg. $NaNO_2$ and the substance given in column 1. In each case enough buffer of p_H 7·6 was added to keep the volume constant. In the anaerobic experiments each tube contained 5 cc. milk, 0·5 cc. 1/5000 methylene blue and the substance given in column 1.

Substance added	Time of aeration	Nitrite remaining unoxidised	Reduction time of methylene blue
Control	∞	+ + + +	∞
1 mg. hypoxanthine	45 mins.	−	2 mins.
1 mg. xanthine	45 „	+	4 „
0·5 cc. 10 % aldehyde	45 „	+ +	58 „
1 mg. adenine	8 hrs.	+ + +	50 „
1 mg. uric acid	8 „	+ + + +	∞
1 mg. guanine	8 „	+ + + +	∞
1 mg. caffeine	8 „	+ + + +	∞
1 mg. hypoxanthine	1 hr.	−	3 mins.
1 mg. hypoxanthine + 3 mg. uric acid	1 „	+ + +	15 „
1 mg. hypoxanthine + 2 mg. adenine	1 „	+ + + +	68 „
1 mg. hypoxanthine + 2 mg. guanine	1 „	+ + + +	15 „
1 mg. hypoxanthine + 2 mg. caffeine	1 „	+	3 „
0·6 mg. hypoxanthine	1 „	−	2 „
6 mg. hypoxanthine	1 „	+ + + +	16 „
0·2 cc. 10 % aldehyde	2 hrs.	+ + +	50 „
1 cc. 10 % aldehyde	2 „	−	10 „

If the oxidation of nitrites could be explained by the occurrence of a coupled reaction, the components of the system would be xanthine oxidase, substrate, nitrite and molecular oxygen. The system proved however not to be quite so simple, because, although whenever milk was used the nitrite was always oxidised, when the caseinogen-enzyme preparation described by Dixon and Thurlow was used, either with hypoxanthine or aldehyde, the nitrite was

never oxidised. There must therefore be present in milk some necessary component of the system not present in the enzyme preparation. It was thought that this might be the fat, which is removed from the caseinogen-enzyme preparation by extraction with ether. Dixon and Thurlow have shown that fat accelerates the oxidation of hypoxanthine, and it was thought that the oxidation of nitrites might be so slowed up by the removal of fat that no change in nitrite concentration could be observed within the time of an ordinary experiment. Boiled cream was therefore added to the caseinogen-enzyme preparation, but again no oxidation of nitrite occurred. It was therefore obvious that the fat was not the required factor.

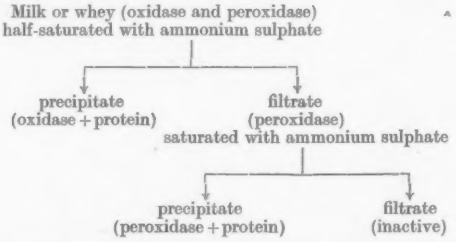
According to Haas and Lee when milk is coagulated with rennin and the whey half-saturated with ammonium sulphate, the precipitate obtained can, in the presence of aldehyde, oxidise nitrites, whereas the filtrate cannot. The filtrate would of course contain no oxidase, because this is precipitated by half-saturation with ammonium sulphate. According to the experiments already given showing that xanthine oxidase is connected with the nitrite reaction, a filtrate containing no oxidase could not be expected to oxidise nitrites. On the other hand when the precipitate was carefully washed with half-saturated ammonium sulphate until it was free from all traces of the filtrate, it behaved just like the enzyme preparation—oxidising hypoxanthine and aldehyde but not nitrites. The only difference between this procedure with the precipitate obtained by half-saturation with ammonium sulphate, and the procedure of Haas and Lee was the thorough washing. The obvious conclusion was that the filtrate contained the unknown nitrite-oxidising factor, which had been separated from the xanthine oxidase by the precipitation, so that neither precipitate nor filtrate caused the oxidation of nitrites. The recombination of the two should again complete the system, and give the same results as milk. This was actually found to be the case, as is shown in the following experiment. The filtrate containing the nitrite-oxidising factor was then saturated with ammonium sulphate and the factor was found to be precipitated completely. Repeating this experiment, using aldehyde instead of hypoxanthine, gives exactly the same results. Typical experiments are given below.

Each tube contained 0·05 mg. $NaNO_2$ + 0·4 mg. Hx + the substance given in column 1. The tubes were aerated at room temperature for 3 hrs.

Substance added	Nitrite remaining unoxidised
5 cc. half-saturated filtrate + 5 cc. 7·6 buffer	+ + + +
5 cc. precipitate obtained by half-saturation + 5 cc. filtrate	+
5 cc. precipitate obtained by half-saturation + 5 cc. buffer	+ + + +
5 cc. saturated filtrate + 5 cc. oxidase preparation	+ + + +
5 cc. precipitate obtained by saturation + 5 cc. oxidase preparation ...	−
5 cc. precipitate obtained by saturation + 5 cc. buffer	+ + + +
5 cc. oxidase preparation + 5 cc. buffer	+ + + +

It can be seen from these experiments that oxidation of nitrite occurs only when the nitrite-oxidising factor is added to the xanthine oxidase.

The question then arises as to the nature of this factor which, in the presence of molecular oxygen, xanthine oxidase and one of its substrates, can bring about the oxidation of nitrites. Haas and Lee consider the possibility of its being peroxidase, but discard it because the filtrate obtained from whey half-saturated with ammonium sulphate gives a peroxidase test but will not oxidise nitrites. It has just been shown that the combination of this filtrate and the precipitate obtained by half-saturation with ammonium sulphate oxidises nitrites, and that it is the addition of the peroxidase-containing filtrate to the xanthine oxidase which brings about the oxidation. The filtrate from whey or milk completely saturated with ammonium sulphate does not give the peroxidase test. Neither does it, when added to xanthine oxidase and hypoxanthine, cause the oxidation of nitrites. In no case has it been possible to get a solution which contained the milk peroxidase and which, when added to xanthine oxidase and its substrate, failed to oxidise nitrite. Nor, on the other hand, has it been possible to get a solution which did not give the peroxidase test but did contain the nitrite oxidising factor. Both the milk peroxidase and the nitrite-oxidising factor are destroyed by boiling but neither is destroyed by acid, so that it seems more than probable that the unknown factor is peroxidase. The following scheme makes clear the separation of xanthine oxidase from peroxidase.



It has not been possible to substitute a vegetable peroxidase in the system. Preparations of the peroxidases of horseradish, malt and potato were tried, but they all contain reducing substances which reduce nitrate with such alacrity that it is impossible to test for the disappearance of nitrite. The artificial peroxidases of Dony-Henault [1908] were tried. That made from ferrous sulphate reduced nitrates and was therefore of no use. The other, made from manganese formate, when substituted for milk peroxidase, did not bring about the oxidation of nitrites.

An animal peroxidase was then made in the following way. Ox liver was perfused until as much blood as possible had been washed away. It was then extracted with water and an equal volume of alcohol added to dissolve the remaining haemoglobin. The precipitate after repeated washing with 50 % alcohol was stirred up in water. The method of Kasanski [1912] for removing catalase by pyrogallol was tried, but it was found difficult afterwards to obtain the peroxidase free from pyrogallol, the presence of which renders impossible

any comparison of pink colours obtained with the Griess-Ilosvay reagent. Owing to the presence of catalase it was impossible to get a negative test, *i.e.* to get all the nitrite oxidised, but in every case there was a distinct difference between the tube containing hypoxanthine and the control. An alkaline phosphate extract of washed ox muscle, which gave a faintly positive peroxidase test, was also tried a number of times. The results of these experiments are given below.

5 cc. liver peroxidase + 5 cc. oxidase preparation	+ 0·05 mg. $NaNO_2$		+ 0·3 mg. Hx	+ +
5 cc. liver peroxidase + 5 cc. oxidase preparation	,,	,,	—	+ +
5 cc. muscle extract + 5 cc. oxidase preparation	,,	,,	+ 0·3 mg. Hx	+ +
5 cc. muscle extract + 5 cc. oxidase preparation	,,	,,	—	+ + + +

These experiments show that an animal peroxidase can be substituted for the nitrite-oxidising factor and that therefore the latter must be peroxidase.

The next question to be considered is the rôle played by the peroxidase in bringing about the oxidation of nitrites in the presence of the xanthine oxidase and one of its substrates. Haas and Lee state that itate causes aldehyde in the presence of molecular oxygen to form aldehyde peroxide, the oxygen of which, when activated by the peroxidase, oxidises nitrite. They were able to get a test for peroxide when milk containing aldehyde was aerated. The activation of the aldehyde, however, as has been shown in the preceding experiments, is not due to an unknown factor, but to the milk oxidase, since any substrate of that enzyme can take the place of the aldehyde in bringing about the oxidation of nitrite. Therefore, if peroxide is formed it must be in the course of the oxidation brought about by the xanthine oxidase. The system would then be as follows:

1. Xanthine oxidase + substrate (Hx, X, or Aldehyde) + O_2 → peroxide
2. Peroxide + nitrite-oxidising factor (peroxidase) + nitrite → nitrate

Haas and Lee observed that nitrites were oxidised by hydrogen peroxide and milk peroxidase, but not by hydrogen peroxide alone. These experiments have been confirmed and the same results have been obtained with a system composed of milk peroxidase, nitrite and hydrogen peroxide as with a system composed of milk peroxidase, nitrite, xanthine oxidase and hypoxanthine or aldehyde. Since the second of the reactions given above is thus shown to occur, it remains to show that the first also occurs. Experiments were carried out to determine whether peroxide is formed in the course of oxidation of hypoxanthine, xanthine and aldehyde by molecular oxygen. Several tests for peroxide were used. Tincture of guaiacum was made up fresh each day and a water extract of horseradish scrapings, which had first been soaked in alcohol and dried, was added when the test for peroxide was made in order to supply the necessary peroxidase. Another very sensitive test is that suggested by Kaiser and McMaster [1908], a mixture of solutions of ferric chloride and potassium ferricyanide. Titanium sulphate was used in several experiments, but the yellow colour caused by the presence of peroxide was obscured to a large extent by the precipitated caseinogen so that it was less easy to detect small amounts of peroxide with this reagent than with guaiacum or the combination

of ferric salts. The benzidine reagent could not be made to work in the presence of the caseinogen-enzyme preparation. A large number of experiments have been done in which a solution of caseinogen-enzyme preparation and hypoxanthine, xanthine or aldehyde has been aerated and tested at intervals for the presence of peroxide. Typical results are shown in Table II.

Table II.

In each experiment to 20 cc. caseinogen-enzyme preparation dissolved in p_H 7·6 phosphate buffer was added 1 cc. of substrate. The results are indicated by an arbitrary standard assumed for each test. The separate experiments are not comparable as far as the relation between time and amount of peroxide present is concerned, because, since various caseinogen-enzyme preparations were used, the activity of the enzyme was not the same in all cases.

Substrate	Test used	Time			Amount of peroxide present
Hypoxanthine	Guaiacum + horse-radish peroxidase	0			− (became blue on standing 10 mins.)
		10	mins.		+ + + +
		20	,,		+ + + +
		40	,,		+
		120	,,		−
Adding more hypoxanthine		20	,,	after addition	+
Hypoxanthine	$FeCl_3 + K_3Fe(CN)_6$	0			−
		20	,,		+
		60	,,		+ +
		90	,,		+
Hypoxanthine	Titanium sulphate	0			− (became faintly yellow on standing 15 mins.)
		20	,,		+
		60	,,		+
		90	,,		−
Xanthine	Guaiacum + horse-radish peroxidase	0			− (became blue on standing 10 mins.)
		10	,,		+ + +
		20	,,		+ +
		40	,,		< +
		60	,,		−
Adding more xanthine		20	,,	after addition	+
Xanthine	$FeCl_3 + K_3Fe(CN)_6$	0			− (became blue on standing 20 mins.)
		20	,,		+
		40	,,		+
		60	,,		+
Aldehyde	Guaiacum + horse-radish peroxidase	0			−
		15	,,		< +
		30	,,		+ +
		45	,,		+ +
		60	,,		−

These results have been obtained so many times that there seems to be no doubt that peroxide is formed in the oxidation of hypoxanthine, xanthine and aldehyde in the presence of molecular oxygen. The fact that the test is negative at the end of the reaction shows that the peroxide must be used up during the course of the reaction. This is explained by the observation of Wieland [1921] that hydrogen peroxide and various other peroxides act as hydrogen acceptors for activated hydrogen, forming water.

The destruction of peroxidase by peroxide was observed by Schellhase [1908]. If peroxide were being formed, one would expect to find the peroxidase

disappearing as oxidation occurred. The gradual destruction of the peroxidase during the course of oxidation of aldehyde in molecular oxygen was observed by Haas and Lee and has been confirmed by my own experiments. I have found that peroxidase is also destroyed during the course of oxidation of hypoxanthine by molecular oxygen. The xanthine oxidase is also destroyed during the course of the reaction with molecular oxygen but not in the anaerobic oxidation.

Still further evidence for the belief that a peroxide is formed in the aerobic oxidation of hypoxanthine, xanthine and aldehyde, and that it is the activated oxygen of this peroxide which brings about the oxidation of nitrite, is afforded by the results obtained when these substances are oxidised anaerobically. In anaerobic oxidations methylene blue takes the place of molecular oxygen as hydrogen acceptor so that a peroxide could not be formed. According to the theory advanced earlier in the paper nitrite should not be oxidised. The following experiment shows that in the anaerobic oxidation of hypoxanthine the oxidation of nitrite does not occur.

Contents of vacuum tube	Reduction time of methylene blue
5 cc. diluted milk + 0·5 cc. methylene blue + 0·2 mg. Hx + 0·1 cc. H_2O	5·5 mins.
5 cc. diluted milk + 0·5 cc. methylene blue + 0·2 mg. Hx + 0·1 mg. $NaNO_2$	6 ,,

If the nitrite were oxidised, the reduction time of methylene blue should be decreased, whereas the reduction time with and without nitrite is the same.

Finally, the results obtained with catalase show definitely that hydrogen peroxide is formed. The work of various authors has shown that in a system containing both peroxidase and catalase the hydrogen peroxide present distributes itself between the two. Therefore if it is really hydrogen peroxide that is being utilised by the peroxidase to bring about the oxidation of nitrites, the reaction ought to be retarded by catalase. Catalase was made according to the method of Batelli and Stern [1904], and the solution of it used was adjusted to p_H 7·6.

Contents of tubes	Nitrite remaining unoxidised
3 cc. oxidase preparation + 3 cc. peroxidase + 4 cc. catalase + 0·05 mg. $NaNO_2$ + 0·8 mg. Hx	+ +
3 cc. oxidase preparation + 3 cc. peroxidase + 4 cc. buffer p_H 7·6 + 0·05 mg. $NaNO_2$ + 0·8 mg. Hx	–

It can be seen that the reaction is inhibited to a marked degree by catalase. There seems to be no doubt therefore that in the course of aerobic oxidation of hypoxanthine, xanthine and aldehyde, hydrogen peroxide is formed.

The problem was now attacked from a different point of view. It has been shown that the oxidation of nitrite depends, not on the presence of peroxidase and a specific oxidisable substance like aldehyde, but on the presence of peroxidase and hydrogen peroxide. The hydrogen peroxide may be produced during the course of oxidation if the entire system is being considered, or it may be added to the peroxidase and nitrite. It might then be argued that if oxidising systems are at all comparable with one another it should be possible

to substitute for the xanthine oxidase and its substrate other oxidising systems, such as another oxidising enzyme and its substrate, or a substance with a readily oxidisable group like the —SH compounds. Succinic oxidase and its substrate sodium succinate were substituted in the system for xanthine oxidase and hypoxanthine. The succinic oxidase was made by Ohlsson's [1921] method. The following is a typical experiment:

Contents of tubes				Nitrite remaining unoxidised
5 cc. succinic oxidase + 5 cc. milk peroxidase	+ 0·05 mg. $NaNO_2$		+ 2·5 mg. sodium succinate	+
5 cc. succinic oxidase + 5 cc. 7·6 buffer	„	„	„ „	+ +
5 cc. succinic oxidase + 5 cc. milk peroxidase	„	„	—	+ + + +

This shows that succinic oxidase and sodium succinate behave like xanthine oxidase and hypoxanthine in the system. The succinic oxidase preparation gave a faint peroxidase test, which accounts for the fact that some nitrite is oxidised without the addition of milk peroxidase.

A solution of succinic oxidase and sodium succinate was aerated and tested without success for peroxide, probably because the peroxide is formed very much more slowly than in the case of hypoxanthine and is used as an oxidising agent as rapidly as it is formed. This is substantiated by the fact that the period of aeration of the succinic system must be about six times as long as that of the xanthine oxidase system with hypoxanthine before the same quantity of nitrite can be oxidised. The succinic oxidase preparation contains a large amount of catalase, and therefore no inhibition was obtained by the addition of more catalase. This result agrees with the curve obtained by Chodat and Pasmanik [1907] for the partition of hydrogen peroxide between peroxidase and catalase. They showed that the addition of catalase beyond a certain point caused no further inhibition of the peroxidase reaction.

The other type of oxidising system, namely that which does not require activation by an enzyme, was then studied. Xanthine oxidase and its substrate were replaced by cysteine, so that the system was now composed of cysteine, peroxidase and nitrite.

Contents of tubes	Nitrite remaining unoxidised
5 cc. cysteine + 5 cc. 7·6 buffer + 0·05 mg. $NaNO_2$	+ + + +
5 cc. peroxidase + 5 cc. buffer + 0·05 mg. $NaNO_2$	+ + + +
5 cc. cysteine + 5 cc. peroxidase + 0·05 mg. $NaNO_2$	–

Thus nitrite is oxidised when cysteine oxidises in the presence of peroxidase, and the inference can be made that during the oxidation of cysteine, peroxide is formed. Professor Hopkins (unpublished experiments) working with the titanium sulphate test was unable to show the presence of peroxide in oxidising cysteine solutions. This can be explained by the observation of Abderhalden and Wertheimer [1923] that cysteine is instantaneously oxidised by added hydrogen peroxide, so that if hydrogen peroxide were formed during the

oxidation of cysteine it would be removed from the solution as soon as produced. Thus the quantity of peroxide in the solution at any instant would be too small to give a positive test. The effect of catalase was then tried but with a negative result; that is, catalase does not inhibit the oxidation of nitrites in the cysteine system. This might be considered as evidence for the formation of an organic peroxide by cysteine. On the other hand it may be explained by the assumption that the peroxidase acts upon very small quantities of hydrogen peroxide more rapidly than does catalase, so that the effect of catalase is so small as to be unobserved.

Further evidence for the formation of peroxide in the oxidising cysteine solution is the fact that during the oxidation peroxidase is destroyed.

Finally, glutathione was used instead of cysteine.

Contents of tubes	Nitrite remaining unoxidised
2·5 cc. glutathione (25 mg.) + 2·5 cc. 7·6 buffer + 0·025 mg. $NaNO_2$	+ + + +
2·5 cc. glutathione (25 mg.) + 2·5 cc. peroxidase + 0·025 mg. $NaNO_2$	+

The above experiment shows that the glutathione system is similar to the others that have already been considered, in that during the course of oxidation of glutathione in the presence of peroxidase nitrite is oxidised, and therefore a peroxide must be formed during the oxidation.

Discussion.

The fact that a peroxide is formed during the oxidation of hypoxanthine, xanthine and aldehyde has been established above, and since such systems as oxidising cysteine, oxidising glutathione and oxidising succinate behave in the same way in bringing about the oxidation of nitrite when peroxidase is added, there is little doubt that in these systems also peroxide is formed. The question arises as to the nature of the peroxide. Is it an organic peroxide or hydrogen peroxide? Bach [1913] believed in the formation of an organic peroxide in some oxidations and of hydrogen peroxide in others. His experimental evidence [1905] for the formation of an organic peroxide was the fact that when he added catalase to a system composed of plant oxygenase, peroxidase and pyrogallol, the oxidation of the pyrogallol was not inhibited. Therefore since catalase destroys hydrogen peroxide but not organic peroxides, that formed by the oxygenase must be organic. Bach [1905] showed, however, that pyrogallol destroys catalase so that in such a system no inhibition could be expected. There seems therefore to have been no evidence that an organic peroxide was formed during the course of such oxidations. Haas and Lee suggest that the peroxide formed in the oxidation of aldehyde is organic, but this view is not based on experimental evidence. There are strong reasons, however, for believing that the peroxide concerned is hydrogen peroxide. In the first place it seems unlikely chemically that compounds such as hypoxanthine would form peroxides. No compounds of such a type are known. Secondly, the explanation of the activity of the enzyme is far more simple if it is assumed that hydrogen peroxide is directly formed. The enzyme can then

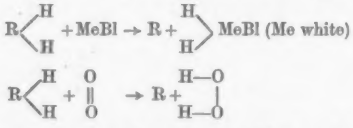
be considered as producing active hydrogen, a point of view which is generally assumed for enzymes of this type, and which makes the oxidation of hypoxanthine and aldehyde comparable to the oxidation of succinic acid. A general consideration of those systems which produce active hydrogen, and hence reduce methylene blue, shows that in every case the active hydrogen produced converts molecular oxygen to hydrogen peroxide [see Traube, 1893]. Armstrong and Colgate [1913] refer to a number of such systems, giving as typical examples:

1. The cathode in the electrolysis of dilute solutions of electrolytes. Any oxygen present is reduced to hydrogen peroxide by the active hydrogen produced.

2. When metals dissolve in acids, active hydrogen is produced which forms hydrogen peroxide with molecular oxygen.

3. Autoxidisable substances such as indigo white and hydrazobenzene during oxidation act as hydrogen donators, and the active hydrogen thus produced forms hydrogen peroxide with molecular oxygen.

Many other cases are known. In any system producing active hydrogen it would seem, then, that either of the following reactions might occur.



Thirdly, as has already been mentioned, milk peroxidase acts upon hydrogen peroxide to bring about the oxidation of nitrites. Fourthly, that it is hydrogen peroxide which is connected with the nitrite oxidation in the system of oxidising hypoxanthine or aldehyde is shown by the inhibition by catalase. Bach and Chodat [1903] have shown that catalase does not decompose organic peroxides. Fifthly, Kastle and Loevenhart [1901] have shown that organic peroxides blue guaiacum directly without peroxidase, whereas hydrogen peroxide blues guaiacum only in the presence of peroxidase. A solution of oxidising hypoxanthine does not cause the faintest blueing of guaiacum until peroxidase is added, whereupon, as already stated, a deep blue appears. Sixthly, it has been shown that succinic acid oxidising to fumaric acid behaves in a manner exactly similar to oxidising hypoxanthine. A consideration of the formulae of succinic and fumaric acids makes the belief in the formation of an organic peroxide during this oxidation untenable; for while it is just possible to view hypoxanthine as a peroxide, it is inconceivable that succinic acid can do anything but lose two hydrogen atoms, which with molecular oxygen would form hydrogen peroxide.

The peroxides in the cysteine and glutathione systems remain for consideration. The reason for assuming the formation of hydrogen peroxide rather than of an organic peroxide in the case of the —SH compounds is only that such a view makes it possible to explain the mechanism of their oxidation in

a manner more nearly comparable to that involved in the other oxidising systems which have been considered. It is impossible at the moment, however, as far as the —SH compounds are concerned, to make a definite assertion as to the nature of the peroxide formed. The case, however, for the direct formation of hydrogen peroxide rather than of an organic peroxide in the oxidation of hypoxanthine, xanthine, aldehyde and succinic acid seems well established.

We now come to a theoretical consideration of the results obtained. Bach maintained that a complete oxidising system consisted of two enzymes. The first was an easily oxidisable body which, with molecular oxygen, formed a peroxide and which he called oxygenase. The second was a peroxidase which acted upon the peroxide formed by the first enzyme, and the oxygen thus activated could bring about the oxidation of some other substance. Because of its properties, Bach believed the oxygenase to be an enzyme, although this point has been disputed by other workers. The Schardinger enzyme was, according to Bach, part of a quite different system. By means of water it reduced methylene blue in the presence of aldehyde, and was therefore called a reducing enzyme. As the same reactions have been shown by Morgan, Stewart and Hopkins [1922] to occur with molecular oxygen as acceptor as with methylene blue, the Schardinger enzyme, or rather aldehyde-oxidising enzyme (since in milk, as has been shown by Dixon and Thurlow, it is probably the same as the xanthine oxidase), can be considered as an oxidising enzyme. The Schardinger enzyme + aldehyde could then be considered as Bach's oxygenase except that the substance upon which the enzyme acts and not the enzyme itself would form the peroxide. Such a view would simplify the consideration of oxidations, as Bach's hydrolytic oxidation-reduction system could be thought of as comparable to his ordinary oxidising system. The experimental facts already given completely bear out such a conception, and explain why in some cases oxygenase seemed to behave like an enzyme, while in others it did not. Considering the oxygenase firstly from Bach's point of view that it is an easily oxidisable substance forming a peroxide, I have shown that in the oxidation of cysteine and glutathione, both easily oxidisable substances, a peroxide must be formed, because when the second part of Bach's system is added, namely peroxidase and another oxidisable substance, oxidation of the latter occurs. Cysteine and glutathione are therefore oxygenases, but cannot be considered as enzymes. But Bach's observation that in many cases the oxygenase is thermolabile and therefore behaves like an enzyme can be explained by the fact that oxygenase may not always be a single substance. In some systems it may be an oxidising enzyme and its substrate such as the xanthine oxidase and hypoxanthine, the aldehyde enzyme and aldehyde, or the succinic enzyme and succinic acid. I have shown that all these systems form peroxide, the oxygen of which in the presence of peroxidase can be used for further oxidations. Onslow [1920] has shown that in plants a catechol derivative and an oxidising enzyme form an oxygenase. She, however, refers to the enzyme as oxygenase, whereas by Bach's

definition the term oxygenase includes both enzyme and peroxide-forming substance.

The only difference in the oxidising systems which I have shown to be oxygenases is that in some cases the oxidation consists of a loss of hydrogen and in others of an addition of oxygen. In the latter case it is necessary to suppose water as a reacting part of the system. The essential mechanism involved, however, can be assumed on Wieland's theory of hydrogen activation to be the same in all cases. Morgan, Stewart and Hopkins suggest that hypoxanthine adds on water to form a hydrate, the hydrogen of which is activated in Wieland's sense by the enzyme and is transported, if the oxidation be anaerobic, to methylene blue, or, if the oxidation be aerobic, to molecular oxygen. I have shown that in the latter case hydrogen peroxide is formed. It might be assumed that the oxidation of —SH compounds is similar to the oxidation of the hypoxanthine hydrate. Warburg claims that —SH compounds can oxidise only in the presence of iron. The iron might be considered as activating the —SH group, thus rendering its hydrogen labile. Mr D. C. Harrison (unpublished experiments) has recently obtained some results which make this view highly probable. The activated hydrogen would reduce methylene blue or with molecular oxygen would form hydrogen peroxide. The action of iron on the —SH compounds would on such a view be analogous to that of the oxidising enzymes on their substrates; and the conception of oxidising systems would be somewhat simpler.

Summary.

1. Hypoxanthine, xanthine and adenine can replace aldehyde in the nitrite-oxidising system in milk, termed itate by Haas and Hill.

2. The caseinogen preparation of xanthine oxidase + one of its substrates will not bring about the oxidation of added nitrite, but will do so if milk peroxidase is added. The complete system consists of xanthine oxidase; hypoxanthine, xanthine or aldehyde; milk peroxidase and nitrite.

3. The peroxidase of liver has been substituted for the milk peroxidase in the nitrite-oxidising system.

4. Peroxide is formed in considerable quantities when hypoxanthine, xanthine or aldehyde is oxidised by the xanthine oxidase in the presence of molecular oxygen.

5. The oxidation of nitrite in the presence of milk, hypoxanthine and molecular oxygen is retarded by catalase.

6. Nitrite is oxidised by the system succinic oxidase, succinic acid, animal peroxidase and molecular oxygen.

7. Nitrite is also oxidised during the autoxidation of the —SH compounds cysteine and glutathione, provided milk peroxidase is present.

8. These results lead to the conclusion that peroxide is formed during the aerobic oxidation of succinic acid, cysteine and glutathione.

9. In the cases of hypoxanthine, xanthine, aldehyde and succinic acid hydrogen peroxide is formed directly by the addition of active hydrogen to the oxygen molecule.

10. The systems xanthine oxidase + any one of its substrates, succinic oxidase + succinic acid, cysteine and glutathione are shown to be oxygenases as defined by Bach.

11. A preparation of animal peroxidase suitable for general use can be obtained by saturating with ammonium sulphate the filtrate obtained by half-saturating milk with ammonium sulphate.

The author wishes to express her sincere thanks to Sir F. G. Hopkins for his continued interest and encouragement during the course of this work.

She is also extremely grateful to Mr M. Dixon for much helpful advice and invaluable criticism.

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XXVI. STUDIES ON THE NATURE OF THE PROCESS OF GERMINATION.

A NEW METHOD FOR THE DETERMINATION OF PROTEINS BY MEANS OF ADSORPTION APPLIED TO THE DECOMPOSITION OF PROTEINS IN GERMINATING PEA SEEDS.

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Earlier publications dealing with the question of decomposition of proteins in germinating seeds, have not given adequate consideration to proteolytic hydrolysis accompanying the extraction process.

Osborne has pointed out that a short extraction at a normal temperature, even if incomplete, yields more satisfactory results than prolonged extraction with heat because in the latter case it is impossible to avoid changes resulting from fermentation.

It is for this reason that the figures given in earlier publications relating to the proportion of protein N to amino N require to be reconsidered. As we are dealing only with proteins which are soluble in neutral solutions of sodium chloride (globulins and albumins) it will be best not to attempt a complete extraction but to determine the proportion of protein nitrogen to amino nitrogen in equivalent parts of the maceration liquid.

It will be later shown that it may be possible to obtain approximately absolute figures.

The usual methods for the determination of proteins were also considered to be unsatisfactory.

The method most often applied in Agricultural Chemistry, *i.e.* the determination of the total nitrogen content and multiplication by a certain factor, is of course useless in this case, in which proteins as well as non-proteins (amino acids, etc.) have to be taken into account.

Precipitation with phosphotungstic acid [Abderhalden and Pettibone, 1912] cannot furnish exact data for the degree of protein decomposition. This precipitation process is accompanied by the precipitation of other more or less complicated protein derivatives together with the protein itself.

Similarly the "Stutzer" method [Rosenthaler] (precipitation with copper hydroxide) does not yield accurate results.

Osborne's method [1924] of fractional "salting-out" is, apart from its intrinsic difficulty, quite useless for our purpose, because it fails completely as a quantitative method. (The method was in fact never worked out for this purpose.)

With a view to separating the proteins from other nitrogenous substances, we have tried to adsorb them by suitable adsorption media and afterwards to eluate[1] them.

Adsorption with aluminium hydroxide gel was first tried. As this substance, except in the presence of special reagents which had to be avoided, is easily soluble in excess of alkali, the less soluble iron hydroxide gel was chosen. It was proved that relatively small quantities of iron hydroxide gel were quite sufficient to adsorb quantitatively all the proteins. The filtrates from this adsorption did not react in the least to Spiegler's or Esbach's reagent, *i.e.* they were absolutely free of proteins.

By the use of weak alkalies it is possible to eluate the proteins almost quantitatively.

An experiment with kaolin (the purified kaolin of May and Baker was used) failed, at first, completely; better results were secured only after slight acidification, *i.e.* acidification of the maceration liquor in slight excess of the optimum for the coagulation of the proteins.

A maceration liquor showed, for example, a p_H of 4·8 (determined with platinum coated electrodes by Luther's method), and was adsorbed only to a slight extent at that concentration. Only when the p_H had been brought to the value of 3·8 by acidification was the adsorption brought practically to completion. The filtrates showed only a weak protein reaction with Spiegler's reagent, after standing for a long time. From the kaolin adsorbate it was possible to eluate the proteins almost quantitatively.

The following figures indicate comparative results with iron hydroxide gel and with kaolin.

100 cc. of maceration liquor contained:

	$Fe(OH)_3$	Kaolin
Total N before adsorption	0·451	0·451
Non-protein N after adsorption	0·038	0·099
Protein N	0·413	0·352
First elution (2 % NaOH) N	0·280	0·288
Second elution (4 % NaOH) N	0·093	0·034
Third elution (6 % NaOH) N	0·020	0·009
Elution (1 + 2 + 3) N	0·393	0·331

It is evident that it is possible to eluate the protein N almost quantitatively. The higher content of non-protein N in the filtrate from the kaolin adsorption is easily explained, because as we have already mentioned the first adsorption by kaolin is by no means absolutely quantitative.

Previous experiments [Abderhalden and Fodor, 1919] and our own experience show that no amino acids are adsorbed when using such reagents.

[1] We are adopting the terminology of Willstätter.

The method of determination is briefly as follows: 100 g. of finely-ground seed-flour is macerated for one hour with 500 cc. of a solution of 10 % sodium chloride at the temperature of the room.

The liquor is then filtered through a battery of folded filter papers and toluene and thymol are added for preservation. In this maceration liquor the nitrogen content is determined by Kjeldahl's process (micro-method of Abderhalden-Fodor).

100 cc. of the maceration liquor is mixed with a corresponding quantity of iron hydroxide gel, completely neutralised and well shaken for half-an-hour. (The iron hydroxide gel was prepared by precipitation of a solution of $FeCl_3$ with the equivalent amount of sodium carbonate and removal of excess CO_2 by boiling.) The resultant precipitate is filtered and thoroughly washed. The filtrate must be absolutely free from proteins; if this is not the case the quantity of iron hydroxide used has been insufficient. The non-protein nitrogen in the filtrate is then determined. To recover the proteins, the residuum is eluated for 2 hours at first with 2 % NaOH, subsequently with 4 % NaOH and finally with 6 % NaOH. About 300 cc. of alkaline liquor are used for both elution and washing on each occasion. Finally by acidifying up to the optimum of coagulation, the proportion of protein which can be precipitated and the proportion non-precipitable can be determined. Protein which cannot be precipitated by acid can be dialysed and isolated. The difference between the amount of total N in the maceration liquor and the adsorption filtrate indicates the content of protein nitrogen. Lipins are present in such small quantities that they can be ignored.

To obtain approximately absolute figures, it is only necessary to determine the proportion of nitrogen in the flour and the nitrogen content in the maceration liquor and to multiply the protein nitrogen content found in the maceration liquid with the corresponding factor.

Thus we found a total nitrogen content of 0·451 % in the maceration liquid, whereas it contained 0·413 % of protein nitrogen. The nitrogen of the seed-flour used amounts to 3·976 %.

It was therefore necessary to multiply the figure obtained by the factor $\frac{3{\cdot}976}{0{\cdot}451} = 8{\cdot}82$. From this, by multiplying the amount of protein nitrogen by 5·7 (the usual factor for conversion of the nitrogen of Leguminosae into protein) [Grafe, 1914], the protein content is found to amount to 20·76 %.

The alternative method of complete extraction cannot be recommended, because as we have already mentioned, proteolytic hydrolysis is liable to take place under such circumstances.

In the examination of germinated seeds smaller quantities of elution reagent and a shorter period of reaction have been used although we did not recover all the proteins in this way.

This new method of protein determination in plant extracts possesses in our opinion the following advantages:

(1) The proportion of protein nitrogen to non-protein nitrogen is quantitatively determined.

(2) The proteins are purified and can easily be recovered.

(3) Proteolytic hydrolysis is avoided.

(4) The method has the advantage of great rapidity.

Experimental.

The peas, "Prince Albert," supplied by the firm Vilmorin, which contain 3·976 % of nitrogen (air dried after removal of the skin) were used. They were sown in germination boxes and Tel-Aviv dune-sand was used as substratum.

The following summary explains the plan of our experiment:

	Root	Stalk	
Experiment Light 1 (after 3 days)	3–4 cm.	—	
Experiment Light 2 (after 11 days)	Strongly developed	*Ca.* 5 cm.	Cotyledons green
Experiment Dark 1 (after 3 days)	3–4 cm.	—	
Experiment Dark 2 (after 11 days)	Strongly developed	*Ca.* 8 cm.	Cotyledons pale

As we had to work on the problem of chemical processes in the seed itself, the latter had to be completely separated from skins, roots and stalks. The following figures indicate the content of protein N and non-protein N.

In 100 parts of the maceration liquor:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Total N	0·451	0·560	0·648	0·526	0·671
Non-protein N	0·038	0·058	0·143	0·065	0·139
Protein N	0·413	0·502	0·505	0·461	0·532

From these figures the proportion of protein N to non-protein N can easily be calculated.

100 parts of *N* contain:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Protein N	91·57	89·64	77·93	87·62	79·28
Non-protein N	8·43	10·36	22·07	12·38	20·72

This summary shows us that there is a steadily increasing dissociation which has about the same value for normal and for etiolated germinated seeds for equal intervals of time. Previous literature has generally given a figure of 89 : 11 for the proportion protein N : amino N [Adolf Mayer].

This somewhat high content can probably be explained by the fact that proteolytic hydrolysis had occurred during the extraction process.

The following figures of Schulze and Flechsig [1885] for germinated seeds can serve for comparison (the peas were germinated on saw-dust in the dark and the figures relate to the whole plant).

N in % of total N:

	Not germinated	First stage
Protein N	86·44	38·33
Amino N	11·62	30·34
N soluble in ethyl acetate	1·94	11·33

The higher content of amino acids in the first stage may be chiefly ascribed to the fact that all these analyses relate to the whole plant. It is well known,

e.g., that the formation of asparagine is chiefly located in the axial portions of the plant.

Schulze [1878] found in 11-day old lupins in the axial portions 31·81 % asparagine in the cotyledons, only 7·62 % in the dry substance. Priaschnikow obtained similar results. It is confirmed that with regard to the axial portions, the N of the asparagine forms a much higher percentage of the total N than in the cotyledons.

Our experiments showed that not all the protein derivatives are immediately utilised for regeneration, but that a high percentage remains in the seed. Later experiments will enable us to decide whether after the dissociation of protein there is also a qualitative difference between the amino acids immediately used for regeneration and those which at first remain in the seed.

Osborne separated the proteins contained in peas by means of fractional "salting out" in different concentrations. We preferred to distinguish between protein fractions, which can be precipitated and those which cannot be precipitated by acid.

We therefore determined on the first two elutions the proportion between these two fractions. For this purpose we carefully acidified to the optimum of coagulation (*i.e.* methyl red showed a weak reaction), and obtained the following results, expressed as % of protein N in the first two elutions:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Protein precipitable by acid	31·17	44·02	47·14	47·08	57·75
Protein non-precipitable by acid	68·83	55·98	52·86	52·92	42·25

These figures are in no way quantitative, as the proportion was only determined in two elutions, in addition to which, as already mentioned, complete recovery of proteins was not carried out, because of the danger of proteolytic changes. It may be mentioned for the characterisation of these proteins precipitated by acid that *they are quantitatively dispersed in alcohol of* 70–80 %.

We hope to make further investigations of the alcohol sols of the plant globulins.

As it has been suspected that these proteins contain phosphoric fractions [Osborne and Strauss, 1915] this question was also examined. According to our experiments iron hydroxide gel is not suited for this purpose as it liberates the phosphoric acid on its surface.

We therefore left the protein of our maceration liquor to be adsorbed by kaolin and examined it after removal of all the proteins to see whether a difference could be observed in the content of phosphoric acid before and after the adsorption.

	% P		
	Non-germinated	Light 1	Dark 1
Maceration liquor before adsorption	0·07	0·06	0·05
Maceration liquor after adsorption	0·07	0·06	0·04

Hence it follows that there are no phosphoric fractions either in the ungerminated or in the germinated seeds.

SUMMARY.

(1) The method of adsorption with iron hydroxide gel is very well suited for the determination of protein and the derivatives of the protein decomposition process. It enables us to obtain the proteins in a pure state and to characterise them.

(2) The content of non-protein N in germinating seeds increases almost equally during the process of germination, whether the germination takes place in dark or in light.

(3) Only a part of the derivatives obtained at the cost of the proteins is immediately used for regeneration.

(4) In non-germinating seeds there is a proportion of 30 : 70 of protein precipitable by acid to protein not precipitable by acid; this proportion changes in germinating seeds where we find almost equivalent quantities of the two kinds of protein. (At the same time there is a loss of total protein N.)

(5) The protein precipitated by acid is dispersed by alcohol of 70–80 %.

(6) Neither in non-germinating seeds nor during germination are there any phosphoproteins.

We have to thank Miss S. Beham for her help in the analytical part of the work.

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XXVII. GLUTATHIONE.

THE OCCURRENCE AND QUANTITATIVE ESTIMATION OF GLUTATHIONE IN TISSUES.

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THE sole information available as to the glutathione content of various tissues is based upon the amounts actually isolated as described by Hopkins [1921] in his original paper.

The figures are as follows:

Yeast. 0·1–0·15 g. per kg.; 0·01–0·015 %.

Muscle. Approximately the same.

Liver. Undoubtedly richer, but yield not recorded.

Professor Hopkins informs me that from yeast later yields of the dipeptide have been greater. In one case 14 g. were isolated from 50 kg. of yeast, a yield of 0·03 % approximately.

As the method of isolation is such that fairly considerable losses are bound to occur, it is obvious that the above figures are of the right order only.

An attempt was therefore made to estimate, more directly and quantitatively, the amount of glutathione in various animal tissues.

EXPERIMENTAL.

The method adopted was as follows. A known weight of the tissue is ground in a mortar with sand and 10 % trichloroacetic acid. The mass so obtained is filtered on a small Buchner funnel and re-extracted twice with further amounts of 10 % trichloroacetic acid.

It was found by the application of the nitroprusside test that this was sufficient to extract all the soluble —SH groups of the tissues.

The use of trichloroacetic acid has the following advantages:

(i) A clear protein-free filtrate is obtained.

(ii) It does not interfere with the subsequent iodine titration.

(iii) Oxidation of the —SH groups during the extraction is prevented owing to the acidity of the solution.

(iv) Glutathione is of course not precipitated by this reagent.

The clear extracts so obtained are titrated with $N/100$ iodine solution, using sodium nitroprusside as an external indicator. From the equation

$$2R.SH + I_2 = R.S.S.R + 2HI$$

1 cc. $N/100$ $I_2 \equiv 2{\cdot}5$ mg. reduced glutathione.

The method has been controlled as far as possible in the following ways. The following substances, which might be present in an extract of tissue prepared as described and which might also react with iodine under such conditions, have been found not to interfere with the estimation:

Urea, Uric Acid, Creatinine.
Glucose. Fructose.

Known amounts of reduced glutathione added to the tissue pulp were satisfactorily estimated.

Estimations have also been made of the sulphate sulphur and total sulphur contained in tissue extracts. The difference gives the soluble organic sulphur. This amount has been found to correspond well with the value obtained for the sulphur present as —SH groups by the iodine titration method described. In no case has the sulphur calculated from the iodine titration been greater than the amount of total organic sulphur.

The following table gives some typical results.

Extract	Iodine titration; cc. $N/100$ I_2	Sulphur calculated from iodine titration as mg. $BaSO_4$	Total sulphur mg. $BaSO_4$	Sulphate sulphur mg. $BaSO_4$	Organic sulphur mg. $BaSO_4$
Liver (rabbit)	15·3	35·6	37·3	0·7	36·6
Liver (rabbit)	10·3	24·1	32	5	27
Yeast	9·6	22·4	30	3	27

It follows, therefore, that by the iodine titration of the trichloroacetic acid extract of the tissue, the —SH groups and the —SH groups alone are being estimated. The results obtained by the method are given in the table following. In calculating, the assumption has been made that all the —SH groups present are those of glutathione. This assumption is considered further in a later section.

The following procedure was adopted with animals. They were chloroformed or stunned, and bled to death from the jugular vein. The tissue required was removed as rapidly as possible and weighed. In the case of muscle, preliminary chopping with scissors before grinding with sand, was found advantageous. Cellular organs (liver and kidney) were ground directly with sand.

In the case of yeast the cells were ground with acid, transferred to a beaker and warmed to 60°, and allowed to cool before filtering. Kieselguhr was used if difficulty was experienced in obtaining a clear filtrate.

Tissue	Weight (g.)	cc. $N/100$ I_2	mg. reduced glutathione	%	Remarks
Yeast. A (1)	20	13·6	34·0	0·17	
(2)	10	7·2	18·0	0·18	Fresh baker's yeast.
(3)	5	4·2	10·5	0·21	
(4)	5	3·8	9·5	0·19	
B (5)	5	6·0	15·0	0·30	Samples had dried through keeping.
(6)	5	5·6	14·0	0·28	
Rat. Liver. A (1) ...	8·0	6·08	15·2	0·19	
(2) ...	7·8	5·6	14·0	0·18	Animals starved for 8 hrs. to free liver from glycogen.
B (3) ...	7·5	4·8	12·0	0·16	
(4) ...	9·0	6·10	15·3	0·17	
Rat. Muscle (skeletal) A	6·1	0·86	2·14	0·035	
B	7·0	1·0	2·5	0·035	
C	7·0	0·92	2·3	0·033	
Blood (whole)	—	—	—	—	Completely absent.
Rabbit. Liver A (1) ...	20	19·2	48·0	0·24	
(2) ...	20	20·0	50·0	0·25	Animal starved 16 hrs. to free liver from glycogen.
B (1) ...	20	21·6	54·0	0·27	
(2) ...	20	22·4	56·0	0·28	
Rabbit. Muscle (skeletal)	15	2·4	6·0	0·04	
	15	2·4	6·0	0·04	
	14·8	2·6	6·5	0·044	
Kidney	8·0	5·6	14·0	0·17	Mean value.
Blood (whole)	20 cc.	—	—	—	Complete absence.
Human blood	20 „	—	—	—	Complete absence.
Egg (hen)	—	—	—	—	Absent.

The mean values obtained are summarised below:

		%	Range %
Skeletal muscle	Rat	0·034	
	Rabbit	0·04–0·045	
Liver	Rat	0·18	0·16–0·21
	Rabbit	0·24	0·22–0·35
Yeast (fresh)		0·18	0·15–0·22

The possibility that an equilibrium between the —SH and the —SS— forms of the dipeptide was disturbed during the excision of the tissue and subsequent manipulation (as in the case of lactic acid) was checked as far as possible by adopting a technique similar to that used by Hopkins and Fletcher [1907] in determining the resting minimum for lactic acid in muscle.

The tissue was removed as rapidly as possible after the death of the animal into a tared, ice-cold beaker, weighed and ground under ice-cold trichloroacetic acid in a mortar surrounded by ice. The whole procedure was carried out as far as possible at 0°. No significant differences were observed between the results obtained at 0° and those at ordinary temperatures.

Discussion of Results.

The assumption has been made that the whole of the soluble sulphydryl groups in the tissues are those of glutathione. At the present moment, there is no evidence that cysteïne exists as such free in normal tissues. Extracts made from large quantities of yeast have been concentrated but in no case was cysteine (or cystine) isolated.

There remains the possibility of course that other peptides of a nature similar to glutathione exist, but until such have been definitely isolated it seems justifiable and convenient to express the results as percentage of reduced glutathione.

The close approximation of the values for the organic sulphur and the sulphur calculated from the iodine titration (which estimates —SH groups only) suggests that by far the greater part of the glutathione exists in the tissues in the reduced form. This deduction has been confirmed experimentally as follows. Two equal portions of the same sample of yeast were taken. In one the reduced glutathione was estimated as described. The other was suspended in a phosphate buffer solution at p_H 7·6 in an evacuated tube and incubated at 37° for one hour. The estimation was then carried out in the same manner. No increase in the amount of reduced glutathione was found. The same was the case with liver and muscle.

If, however, oxidised glutathione was added to the sample, a considerable increase in the amount of reduced glutathione was found. The lack of any increase in the experiment was thus due not to the inability of the tissue to reduce the —S—S— to the —SH group, but to the absence of the —S—S— group.

This result is of interest since evidence is accumulating that the —SH group is the active group in the catalytic oxidation processes in which glutathione plays a part.

The liver is undoubtedly the richest organ, and it will be noticed that the organs of the rabbit have a somewhat larger content than the corresponding organs of the rat.

The figures obtained for skeletal muscle (both in the rat and the rabbit) have been exceedingly consistent; those for the liver were variable. The variations in the case of the liver may in part be due to differences in the amount of blood remaining in the organ after bleeding the animal.

In cases where bleeding was poor, and the organ distended with blood, the percentage was always rather lower than usual. The effect of loss of moisture is well shown in the case of yeast; consequently fresh samples were obtained for each determination.

The values obtained by this method of estimation are from 3–5 times greater than the amounts actually isolated.

When the possibilities of loss in the various stages of the separation are considered, the difference may be well accounted for. At one stage alone—precipitation with mercuric sulphate reagent—losses of the order of 40 % have been recorded with cystine [Hopkins, 1921] and a similar loss is probable in the case of glutathione.

Abderhalden and Wertheimer [1923] have estimated colorimetrically the substance reacting with nitroprusside in horse muscle. This, calculated as cysteine, was present to the extent of 100 mg./kg. As glutathione, this on recalculation gives 0·02 %.

The method used by these authors depends on the matching of the nitroprusside colour with a standard mixture of dye-stuffs (Bordeaux red and methylene blue). The nitroprusside colour fades with exceptional rapidity and although, as stated by Abderhalden and Wertheimer, the colour may be made to persist to some extent by the addition of potassium cyanide, the sources of error would appear to be considerable.

It has already been pointed out that no evidence exists for the presence of free cysteine in the tissues of normal animals; and only in cases of cystinuria has the existence of cystine in the tissues been shown.

SUMMARY.

(1) Glutathione in the tissues of normal animals is chiefly in the reduced form (—SH).

(2) The sulphur equivalent to the —SH groups present accounts for the greater part of the soluble "neutral" sulphur of tissue extracts.

(3) The glutathione content of certain tissues has been estimated and values are given.

My thanks are due to Sir F. G. Hopkins, F.R.S., for his encouragement during the progress of this work.

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XXVIII. GLUTATHIONE.

RELATION BETWEEN THE TISSUES AND THE OXIDISED DIPEPTIDE.

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In his original paper on glutathione Hopkins [1921] showed that the hydrogen-ion concentration of the medium had a considerable effect on the velocity of reduction of methylene blue by tissues. This was the case both with fresh tissues and with washed tissues artificially supplied with oxidised glutathione.

He noted that, whereas at reactions slightly on the alkaline side of neutrality the addition of the oxidised dipeptide markedly accelerated the reduction of the dye, alteration of the reaction to the acid side of the neutral point resulted in a retardation of the reduction on the addition of the dipeptide. The explanation was advanced that the —SH groups formed by the reduction of the disulphide form were unable to transfer their hydrogen at an acid reaction, and thus the oxidised glutathione was acting as a competitor with the methylene blue for the available labile hydrogen of the tissues.

In view of the later results of Dixon and Tunnicliffe [1923], who showed that even at p_H 5·0 reduced glutathione was able to reduce methylene blue, it appeared to be of value to continue the investigation of the effect of p_H on the systems described by Hopkins, and Hopkins and Dixon [1922].

Experimental.

Methylene blue technique.

Details of a typical experiment are given below.

Sheep liver was minced and washed six times with distilled water. It was then ground under alcohol, dried and again washed with distilled water. To each of a series of tubes were added 0·5 g. of the tissue, 0·3 cc. of 1/5000 methylene blue solution, and the requisite quantities of oxidised glutathione and phosphate buffer solutions. In each case the total volume was kept the same. The tubes were then evacuated and placed in the water-bath at 37°. The times for decolorisation of the dye were noted and are tabulated below.

Oxidised glutathione mg.	Reduction time (minutes) at p_H 7·6	at p_H 6·2
0	145	245
2	120	210
4	90	180
8	40	122

Similar results have been obtained with both heated and unheated preparations of the muscle of the rabbit, rat, dog and cat. In no case within the limits of p_H examined has there been observed a retarding effect on the addition of oxidised glutathione.

The reactions occurring in the system may be represented as follows:

(i) $T\langle^{H}_{H} + MB \longrightarrow T + MBH_2$

(ii) $T\langle^{H}_{H} + G.S.S.G \longrightarrow T + 2G.SH.$

(iii) $2G.SH + MB \longrightarrow G.S.S.G + MBH_2$

where $T\langle^{H}_{H}$ represents the hydrogen donator of the tissues.

MB and MBH_2 methylene blue and methylene white.
G.S.S.G and G.SH the oxidised and reduced forms of the dipeptide respectively.

The above result shows that the direct reaction (i) is slowed by increasing acidity. The combined reactions (ii) and (iii) proceed at a greater rate than the single direct reaction no matter whether the p_H be on the acid or alkaline side of the neutral point. As might be expected from the results of Dixon and Tunnicliffe [1923] the whole process (i), (ii) and (iii) proceeds more rapidly in alkaline than in acid solutions. Further analysis of the results is rendered somewhat difficult owing to the complicated nature of the system. The fact that the reaction (iii) is slowed by increasing acidity is suggestive that the effect of p_H on the whole system is due to its effect on reaction (iii).

The method throws no light on what is perhaps the most interesting of the reactions—that between the hydrogen donators of the tissue and the oxidised glutathione. An attempt has therefore been made to attack the problem directly.

The method used was as follows. A known weight of the tissue preparation was suspended in a solution of oxidised glutathione at the required p_H and the tube exhausted. After being kept for the necessary time at 37° the tube was opened and the reduced glutathione estimated as described in a previous paper [Tunnicliffe, 1925]. The end point can be determined either by the use of starch, or by using sodium nitroprusside as an external indicator in the usual manner. In the case of heated tissue preparations the end point with starch is fairly sharp, but in the case of the cold washed preparation it is preferable to use the external indicator. The results by the two methods agree fairly closely.

The results of a typical experiment showing the course of reduction of oxidised glutathione by a muscle preparation are given graphically in Fig. 1.

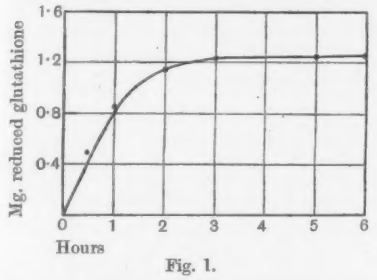

Fig. 1.

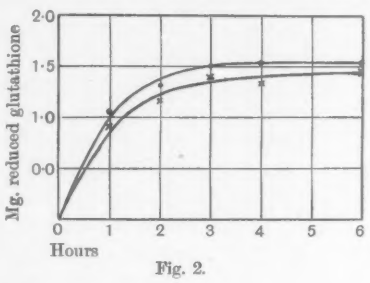

Fig. 2.

Fig. 1. Reduction of oxidised glutathione by muscle.
Thermostable preparation of cat muscle, 0·2 g.
Oxidised glutathione, 8 mg.
Phosphate buffer solution, p_H 7·6.

Fig. 2. Reduction of oxidised glutathione by muscle.
—×—×— Washed, heated.
—●—●— Washed, unheated.
Equal weights of washed unheated rabbit muscle were used for each determination in the two series. The muscle in the second (heated) series was plunged for 5 minutes into boiling water before use.
Phosphate buffer solution, p_H 7·6.

A series of tubes was used and estimations made on separate tubes at suitable intervals. It will be observed that the reduction occurs rapidly for the first three hours, and that after this time the rate of the reaction diminishes until after five hours the reaction is complete. The amount of reduced glutathione formed is considerably less than the amount of oxidised glutathione added. This point will be referred to again in a later section of the paper.

In view of the statements of Abderhalden and Wertheimer [1923] that frog muscle can be washed until it shows no power of reducing cystine, and that the reducing power can be restored by the addition of "Kochsaft," or by heating, these points were examined in some detail.

On exposure of various tissue preparations to the air it has frequently been observed that there was a perceptible diminution in the capacity of reducing the oxidised dipeptide; particularly when exposed in thin layers in the moist state. Hopkins and Dixon [1922] also noted this fact. For this reason the tissues have been washed anaerobically by shaking with water in a stoppered cylinder through which nitrogen had been bubbled.

Frog muscle was minced by a special machine to a much finer state of division than can be obtained by chopping with scissors. It was then washed anaerobically with boiled tap water until all traces of haemoglobin were removed, and finally shaken in nitrogen for 4 hours with distilled water and toluene. After drying with alcohol in the usual manner the preparation was found to reduce oxidised glutathione and cystine vigorously.

The effect of heating the tissue preparation for a short time at 100° on the course of the reduction was also examined. The results are shown in Fig. 2 (p. 201). The reaction proceeds somewhat more smoothly in the case of the heated preparation, but within the limits of experimental error the total reduction is the same in both cases. The alteration in permeability of the preparation due to the heating would in all probability account for the greater ease of reduction in the case of thermostable preparations.

As mentioned previously the amount of oxidised glutathione added to the tissue preparation was in excess of the reducing capacity of the tissue used. An experiment was therefore carried out using in each case the same weight of muscle preparation but gradually increasing the amount of oxidised glutathione added. The reaction was allowed to proceed in each case for 6 hours and the reduced glutathione then estimated. In Fig. 3 the abscissae represent the amounts of oxidised dipeptide added and the ordinates the amounts of reduced glutathione formed. The results show clearly that below a certain amount the oxidised glutathione is practically completely reduced; after this point further increase in the amount of the oxidised form leads to no further formation of reduced glutathione. This maximum amount of oxidised glutathione which a given weight of a tissue will reduce is constant within the limit of experimental error for any given preparation, and is to be regarded as a measure of that labile hydrogen of the tissue which is concerned in the reaction

$$T\begin{matrix}H\\H\end{matrix} + G.S.S.G \longrightarrow T + 2G.SH.$$

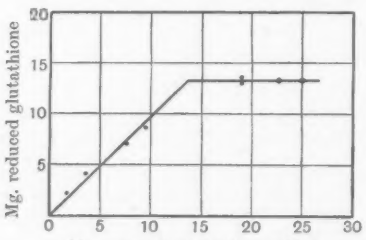

Fig. 3.

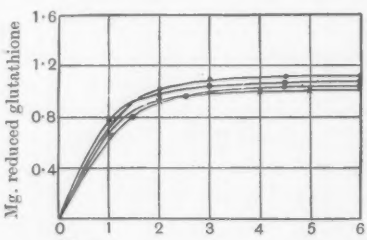

Fig. 4.

Fig. 3. Relation between amount of reduction and amount of oxidised glutathione present. For each determination 2 g. of a thermostable preparation of cat muscle was used, suspended in phosphate buffer solution p_H 7·6.

Fig. 4. Effect of p_H and nature of medium on course of reduction.
—●—Phosphate buffer solution p_H 7·6.
—■— ,, ,, ,, p_H 8·0.
—×— ,, ,, ,, p_H 6·0.
—○— Solution adjusted to p_H 7·6 with dilute Na_2CO_3 solution.

The effect of changes in the nature of the medium in which the tissue was suspended was next examined. The results shown in Fig. 4 may briefly be stated as follows. No significant difference in the rate or amount of reduction

could be detected by altering the p_H or the salts of the medium. Thus there was no difference between the course of reduction in phosphate buffer solution at p_H 7·6, and that in a dilute sodium carbonate solution of approximately the same p_H.

The bearing of the facts above may now be considered in relation to the complete system (Tissue + Oxidised Glutathione + Hydrogen Acceptor). Where methylene blue is the hydrogen acceptor a clear conception of the process can be obtained.

The first point of interest is that the amount of reduced glutathione formed by a given weight of a tissue preparation in contact with excess of the oxidised dipeptide corresponds extremely well with the maximum amount of methylene blue which the same weight of the preparation will reduce. Thus 1 g. of a muscle preparation was found to reduce 6·5 mg. of the dipeptide. The same amount of tissue was found by the method described by Hopkins and Dixon [1922] to reduce between 4·8 and 4·9 cc. of 1/1000 solution of methylene blue. Calculating from the equation $2G.SH + MB = G.S.S.G + MBH_2$ this is equivalent to 6·4 mg. of reduced glutathione.

The result of alterations of hydrogen-ion concentration on the system is also clearly explained. At p_H 7·6 and 6·0, for example, the rate of the reaction $T\begin{smallmatrix}H\\H\end{smallmatrix}$ + G.S.S.G, as shown above, will be the same in both cases. The rate of the final reaction $2G.SH + MB$ will however be considerably less at the lower p_H and consequently the time for reduction of the methylene blue will be greater in the second of the two cases.

When oxygen is the hydrogen acceptor the bearing of the results described is more interesting and at the same time more complex. Calculating, as in the case of methylene blue, the volume of oxygen equivalent to the reduced glutathione formed by 1 g. of a thermostable muscle preparation, the oxygen uptake is found to be of the order of 140–150 $mm.^3$. This figure is of course unaffected by the nature of the medium in which the tissue is suspended, for, as has been pointed out, the amount of reduced glutathione formed is independent of changes in the medium.

The oxygen uptake actually measured in the Barcroft apparatus is in phosphate buffer solution 400 $mm.^3$ per g. of tissue preparation. In Ringer solution or in the absence of phosphate ions the measured uptake is increased to four times this amount, *i.e.* 1600 $mm.^3$ per g. approximately.

There seems no doubt therefore that in addition to the oxidation of those substances which anaerobically are solely connected with the reduction of oxidised glutathione, there must also occur an aerobic oxidation of some other substance associated with the thermostable tissue residue. This additional oxidation occurs in the presence of phosphate ions but to a very much larger extent in their absence. Meyerhof [1923] has recently shown that the oxidation of an unsaturated fatty acid, linolenic acid, by oxygen, is greatly accelerated by the presence of a sulphydryl group which is also undergoing oxidation at

the same time. The oxidation of the linolenic acid in presence of a sulphydryl group is greatly diminished by phosphate ions. Meyerhof used as a sulphydryl compound thioglycollic acid. He also showed that the alcohol-ether extract of thermostable muscle preparations contained an unsaturated acid associated with the lecithin fraction. Through the kindness of Professor Hopkins I am able to state that the oxidation of unsaturated fats and fatty acids, in particular linolenic acid, by oxygen is greatly increased when the reduced form of glutathione is simultaneously undergoing oxidation. Phosphate ions also inhibit this oxidation of the double bonds of linolenic acid in the presence of reduced glutathione.

There seems therefore to be no doubt that the oxidation of the unsaturated fatty acid is the cause of the discrepancy between the oxygen uptake as measured in the Barcroft apparatus, and the uptake calculated from the amount of reduced glutathione which the preparation will form anaerobically.

Meyerhof however appears to regard the unsaturated acid as being the substance responsible for the reduction of the disulphide (dithiodiglycollic acid in his experiments) to the sulphydryl form according to the equation

$$-CH = CH- + \begin{matrix} HOH \\ HOH \end{matrix} + T.SS.T \longrightarrow -CHOH-CHOH- + 2T.SH.$$

This view is at variance with the results which have been described above, and the following experiments were therefore carried out to determine whether there was such a possibility in the case of glutathione. The substances shown in the table below were kept in evacuated tubes in the water-bath at 37° for 20 hours.

Tube	Linolenic acid mg.	Oxidised glutathione mg.	Medium	p_H	Methylene blue 1/5000 cc.
1	20	10	Borate	6·5	0·0
2	20	10	„	6·5	0·3
3	20	10	„	7·8	0·0
4	20	10	„	7·8	0·3
5	20	10	„	8·4	0·0
6	20	10	„	8·4	0·3

Tubes 1, 3 and 5 were opened and the nitroprusside test was found to be negative in each case. There was therefore no reduction of the disulphide form to the sulphydryl form. This was confirmed by the fact that the methylene blue was not reduced in tubes 2, 4 and 6, although there was a strong adsorption of the dye on the surface of the oil droplets which separated out.

It is thus clear that linolenic acid is unable to reduce oxidised glutathione; if such reduction did take place the fact (communicated to me by Professor Hopkins) that the disulphide form causes no increase in the oxidation of the linolenic acid by atmospheric oxygen, would be meaningless.

This has also been confirmed as follows: thermostable residues were extracted for 6 hours in a Soxhlet apparatus with alcohol, ether and acetone in the order described. The total extraction lasted 18 hours and should have

removed thoroughly any fatty acid which was present. Despite this violent treatment the tissue residue still possessed over 50 % of its original capacity for reducing the oxidised dipeptide.

What diminution was observed may well have been due to oxidation of the hydrogen donators of the tissue during the extraction, since ether when boiled tends to form an ether peroxide.

The picture of the aerobic oxidation process suggested previously is thus supported. The linolenic acid is oxidised concurrently with the oxidation of the sulphydryl groups of the glutathione which are established by the reduction of the disulphide form by the hydrogen donators of the tissues. So long as the supply of the donators remains unexhausted there will be a cyclic process involving the alternate reactions

$$\text{(i)}\quad T\begin{matrix}H\\H\end{matrix} + G.S.S.G = T + 2G.SH.$$

$$\text{(ii)}\quad 4G.SH + O_2 = 2G.S.S.G + 2H_2O.$$

Concurrently with reaction (ii) will occur the oxidation of the double bonds of the unsaturated acid. When the hydrogen donators are exhausted reaction (i) ceases, the sulphydryl groups are oxidised, and the system ceases to take up oxygen. With the disappearance of the sulphydryl groups the oxidation of the double bonds of the unsaturated fatty acid ceases.

The effect of alteration of the hydrogen-ion concentration on the aerobic process remains to be considered. At p_H 7·6 the oxidation of reduced glutathione proceeds very rapidly compared with the rate at which the tissue can reduce the oxidised dipeptide. A very low concentration of the sulphydryl form would therefore be expected. This is in agreement with fact, for under the conditions described the nitroprusside reaction is exceedingly faint. The amount of reduced glutathione present was insufficient for estimation by the method described. At p_H 6·0 the rate of oxidation of the reduced glutathione is much slower than at p_H 7·6. As the rate at which the oxidised dipeptide is reduced is unaltered by alteration of p_H a higher concentration of the —SH form is therefore to be expected at the lower p_H, other conditions being the same in the two cases. This is also confirmed experimentally, both by estimation and by means of the intensity of the nitroprusside reaction, particularly in the earlier stages of the aeration of the system.

In cases where the dipeptide is originally present in the reduced form, the conditions will obviously be different from the cases in which it was added in the oxidised form. At a p_H of 7·6 the oxidation of the —SH groups already present will proceed with considerable rapidity and will be practically complete in one hour. There will thus be an initial rapid uptake of oxygen, after which the conditions will approximate closely to those in the system commencing with the glutathione in the —S—S— form. The final total uptake of oxygen will be greater in the case where the glutathione was originally in the —SH form, by an amount of oxygen equivalent to this quantity of —SH groups.

At a more acid reaction, *e.g.* p_H 6·0, the oxidation of the —SH groups already present will be very much slower than at p_H 7·6 and there will thus be a higher concentration of the —SH form at the lower p_H.

The exact effect of the varying concentrations of —SH groups on the velocity of oxygen uptake by the system is difficult to predict; the fact that the disulphide form as shown by Dixon and Tunnicliffe accelerates the oxidation of the reduced form of the dipeptide [1923], and the close relation between the oxidation of the unsaturated fatty acids and that of the —SH groups introduce factors which render such an attempt at present inadvisable. The ultimate solution will rest on a further knowledge of the kinetics of the reaction

$$4G.SH + O_2 = 2G.S.S.G + 2H_2O$$

both in the absence and presence of unsaturated fatty acid linkages.

It is hoped to continue the investigation from this point of view.

Summary.

(1) A study has been made of the reaction between the thermostable tissue residue and oxidised glutathione.

(2) The bearing of the results obtained is discussed with reference to the reduction of methylene blue, and to the uptake of oxygen by tissue residues in the presence of oxidised glutathione.

(3) Linolenic acid was found not to be the substance in the thermostable residue responsible for the reduction of oxidised glutathione.

I am greatly indebted to Sir F. G. Hopkins, F.R.S., for allowing me to use certain of his results, and for his constant interest during the course of the work.

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XXIX. GLUTATHIONE.

SYNTHESIS.

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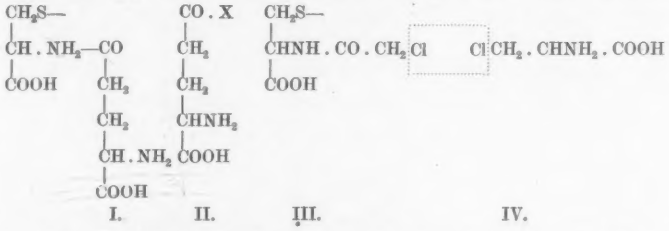
(*Received January 21st, 1925.*)

In a previous paper [1923] the authors, with Mr J. H. Quastel, put forward the view that glutathione possesses the constitution represented by Formula I.



I. II. III. IV.

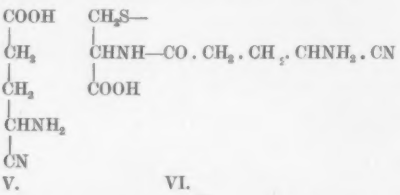
The evidence on which they based that view was derived from a study of the breakdown of the glutathione molecule in various directions. The fact that condensation of glutathione with 2 . 3 . 4-trinitrotoluene (which condenses with primary amine groups) and subsequent hydrolysis of the product yielded free cystine showed that in glutathione the free amino group was that of the glutaminic portion. Confirmation of this was given by treatment with nitrous acid followed by hydrolysis, when α-hydroxyglutaric acid was isolated. Oxidation by hydrogen peroxide gave a compound from which succinic acid was obtained after hydrolysis. This, by analogy with the work of Dakin [1905] on the oxidation of amino acids, was held to indicate that that carboxyl group of glutaminic acid remote from the amino group was involved in the peptide linkage. It was pointed out, however, that the constitution of glutathione could only be regarded as definitely established when a substance of the proposed formula had been synthesised and shown to be identical with the natural product.

The problem presented in the synthesis—that of linking the amino group of cystine with that carboxyl group of glutaminic acid which is remote from

the amino group—resolved itself into the preparation of the appropriate mono-acid halide of glutaminic acid (II) or of some substance which can subsequently be converted to glutaminic acid.

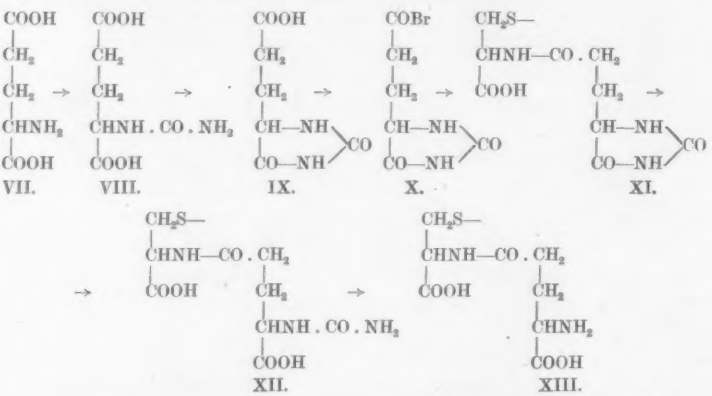
Alternatively, the following synthesis, which, though indirect, would definitely establish the constitution, was considered. Cystine readily condenses with chloroacetyl chloride [Fischer and Suzuki, 1904] giving dichloroacetyl-cystine (III). By elimination of chlorine from this and β-chloroalanine (IV) glutathione should result. This method was abandoned, however, not only because of its tedious nature—it involves the preparation of serine—but also because it was found that the condensation of even simple chloro compounds in the presence of "molecular" silver, sodium, etc., was extremely unsatisfactory.

The preparation of the compound V by Strecker's reaction on the semi-aldehyde of succinic acid presents itself as a solution until it is remembered that after halogenation and coupling of the acid halide to obtain the compound VI, prolonged hydrolysis would be necessary to convert the nitrile group to the required carboxyl group. Such hydrolysis would certainly disrupt the peptide linkage and thus defeat the end in view.



V. VI.

The method finally adopted is shown in the scheme below.



Glutaminic acid (VII) was converted to the hydantoinpropionic acid (IX) by the method of Dakin [1919]. This, on treatment with phosphorus tribromide is converted to the acid bromide (X) which is then coupled with cystine

dimethyl ester hydrochloride [Fischer and Suzuki, 1905] in the ordinary way. The opening of the hydantoin ring presents a problem similar to that encountered by Dakin in his synthesis of β-hydroxyglutaminic acid. Boiling with barium hydroxide, he found, brought about complete decomposition of the bulk of his substance (hydantoin-β-bromopropionic acid). Hydantoin-acrylic acid, on prolonged treatment with the same reagent, gave off ammonia and carbon dioxide with formation of the amino acid, but considerable losses were again experienced. His alternative method of heating in a sealed tube with fuming hydrochloric acid at 130° is obviously inadmissible in the present case owing to the presence of a peptide linkage. Boiling for a short time with calcium hydroxide, however, appeared to open the ring with, probably, the formation of a uramino acid (XII), and with the destruction of only a small amount of cystine. Rohde [1918] has shown that uramino acids, treated with nitrous acid, lose half their nitrogen. She did not, however, identify the products of the reaction. Dakin (private communication) has found that from β-*p*-hydroxyphenyl-α-uraminopropionic acid, under such treatment, tyrosine can be obtained in considerable yield. It is reasonable to suppose that glutaminyl-cystine-uramino acid (XII) would behave in the same way, and accordingly the solution of the substance obtained by the action of calcium hydroxide on di-hydantoinpropionyl-cystine (XI) is treated with nitrous acid in slight excess of the amount required for the removal of one nitrogen atom. The isolation of the dipeptide (XIII) obtained in this reaction is accomplished through the mercuric sulphate compound.

Whilst the substance so obtained agrees with the natural substance in chemical properties and in certain physical properties, it has not the same optical activity. This is most probably due to racemisation during the formation and subsequent opening of the hydantoin ring [Dakin, 1910]. The agreement in all other respects, however, is so close that it is difficult to imagine any difference between the synthetic and natural substances save that of configuration. The differences between the three *possible* constitutions of glutathione are so marked, that did glutathione not possess the constitution proposed, and, consequently, not that of the synthetic product, the two substances must inevitably differ more widely. Especially is this true of the melting point, even though such a criterion is of lessened value when one is dealing with amorphous substances.

Nevertheless, it seemed desirable, since resolution of the racemic peptide appeared a well nigh hopeless task, to attempt a synthesis, which, while yet affording confirmatory evidence of the constitution, should give a substance completely identical with natural glutathione. This object was successfully attained by a direct coupling of cystine and glutaminic acid.

Glutaminic acid was found to react with phosphorus tribromide. The ease with which the product of this reaction was hydrolysed, and its ability to condense with aniline in slightly alkaline solution, showed it to be an acid bromide. Analysis of the anilide gave figures in close agreement with those required for

the hitherto unknown glutaminyl mono-anilide. The reaction of phosphorus tribromide and glutaminic acid had therefore yielded a *mono*-acid bromide. The two-fold object of the synthesis—to confirm the constitution and to obtain a product identical with natural glutathione—demanded that before proceeding further, the constitution of this acid bromide should be definitely established. For this purpose glutaminyl mono-anilide was prepared in sufficient quantity, and use was made of the oxidation method employed in determining the constitution of glutathione itself. It was there argued [Quastel, Stewart and Tunnicliffe, 1923] that the two compounds: $R.NH.CO.CH_2.CH_2.CHNH_2.COOH$ and $R.NH.CO.CHNH_2.CH_2.CH_2.COOH$ should behave differently on oxidation with hydrogen peroxide. Dakin [1905] has shown that α-amino acids, treated in this way, eventually yield fatty acids containing one carbon atom less, glutaminic acid for example, being converted to succinic acid. The first, therefore, should yield $R.NH.CO.CH_2.CH_2.COOH$ which, on hydrolysis, should be broken up to $R.NH_2$ and succinic acid. The second should yield $R.NH.CO.CO.CH_2.CH_2.COOH$ which further oxidation should split up, possibly to succinic acid. At any rate, the complete breakdown should, in this case, take place before hydrolysis. In the particular case of glutaminyl mono-anilide, the problem is simplified by the fact that succinyl mono-anilide is known (see Beilstein's *Handbuch III*, II, 413) so that hydrolysis after oxidation is unnecessary. In actual fact, oxidation of glutaminyl mono-anilide yielded a substance extractable by ether, which was identified by properties, melting point, and analysis, with succinyl mono-anilide. The acid bromide from which it was derived was thus represented by the formula:

$$BrOC.CH_2.CH_2.CHNH_2.COOH.$$

That is, it had the bromine atom attached to the carboxyl group remote from the amino group, and was therefore the one required for the synthesis of a compound having the constitution proposed for glutathione.

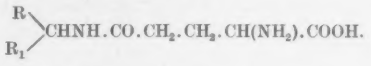
Condensation of the acid bromide with cystine yielded a dipeptide which had all the properties of natural glutathione, and which, on oxidation with hydrogen peroxide behaved in exactly the same way, yielding succinic acid only after hydrolysis of the oxidation product.

The comparison of the physiological properties of the natural and synthetic dipeptides has not yet been fully worked out. It has been observed, however, that like natural glutathione, both the synthetic products restore to a thermostable preparation of dog muscle the power of reducing methylene blue. Further, when either of them is added in "oxidised" (disulphide) form to such a preparation, and is incubated *in vacuo* at 37° for a few minutes, the —S—S— grouping is reduced to —SH.

Both the methods of synthesis involve the coupling of an acid bromide with cystine dimethyl ester. In both of these reactions considerable losses were experienced, so that the final yields of dipeptide were small. Little better, however, is to be expected in such a case where, besides the main

reaction, an acid bromide and an ester are undergoing simultaneous hydrolysis in a slightly alkaline medium.

The methods described in this paper, are, of course, generally applicable to the synthesis of dipeptides of the type:



Experimental.

Synthesis I.

Preparation of the Acid Bromide of Hydantoinpropionic Acid.

The dry, finely powdered hydantoinpropionic acid was covered with excess of phosphorus tribromide, and the mixture was heated on the water-bath for two to three hours in a wide-mouthed flask under a reflux condenser. The yellowish coloured mass was freed from as much as possible of the excess of phosphorus tribromide by decantation, and the remainder was removed by repeated trituration with dry chloroform. It was then transferred to a vacuum desiccator containing sulphuric acid and paraffin wax. The bromine content of the product was estimated by hydrolysis for 20 minutes with excess of a twice normal solution of potassium hydroxide, acidification with nitric acid, and precipitation of the silver bromide with silver nitrate.

0·512 g. of the product gave 0·369 g. of silver bromide. Hence 90 % of the hydantoinpropionic acid had been converted to the acid bromide. No further purification of the crude product was attempted.

Preparation of Di-hydantoinpropionyl-cystine.

5 g. of cystine dimethyl ester hydrochloride, prepared according to the method of Fischer and Suzuki [1905], were dissolved in 50 cc. of ice-cold water giving a clear solution. After further cooling in a freezing mixture (as short as possible since the ester begins to be hydrolysed at once) solid sodium bicarbonate was added until the solution was just alkaline to methyl red. While still in the freezing mixture, the solution was vigorously stirred by a mechanical device, and alternate small portions of the hydantoinpropionyl bromide and solid sodium bicarbonate were added, the solution being kept just alkaline to methyl red, excess being avoided, and 30 % excess over calculated quantity of the acid bromide was added—in the case described, 11 g. At the completion of the reaction the p_H was adjusted to the neutral point (to methyl red). Standing overnight completed the precipitation of unchanged cystine, which was then filtered off. The filtrate contained di-hydantoinpropionyl-cystine along with hydantoinpropionic acid, traces of cystine, and sodium salts. It was acidified with sulphuric acid, and treated

with a little dilute solution of sodium nitrite. Any uncombined cystine was thus destroyed, whereas the di-hydantoinpropionyl-cystine was unattacked owing to the absence of any free amino group from the molecule. The solution was then precipitated with the Hopkins-Cole mercuric sulphate reagent, and, after standing overnight, the heavy white precipitate was filtered at the pump and washed thoroughly with distilled water. Since hydantoinpropionic acid is not precipitated by mercuric sulphate, this precipitate consisted solely of the mercuric sulphate compound of di-hydantoinpropionyl-cystine. It was suspended in water and decomposed by hydrogen sulphide in the usual way. The filtrate was freed from hydrogen sulphide by aeration, and then made alkaline with barium hydroxide. The aeration was then continued until the vivid nitroprusside reaction had disappeared, barium was removed quantitatively with sulphuric acid, and the filtrate was concentrated *in vacuo* to a syrup. Considerable excess of a mixture of one part of absolute alcohol to two parts of dry ether was then added and the sticky product which separated was allowed to stand under the mixture until it became friable. It was filtered off rapidly and dried in a vacuum desiccator containing sulphuric acid, phosphorus pentoxide, and paraffin wax.

The product so obtained, which had a slight yellow colour, was exceedingly soluble in water giving an acid solution. It was insoluble in ether and in acetone. It was precipitated from aqueous solution by mercuric sulphate. The aqueous solution gave no nitroprusside reaction, but after reduction with magnesium and dilute hydrochloric acid an intense reaction was obtained. Heated in a capillary tube, it softened at 140° and melted at 174° (uncorr.).

Nitrogen by Micro-Kjeldahl. Found: N = 15·15 %. Calculated for $C_{18}H_{24}O_{10}N_6S_2$: N = 15·32 %.

Amino Nitrogen by the method of Van Slyke. Traces only of nitrogen were evolved. After hydrolysis, however, a qualitative test showed the presence of free primary amino groups.

Sulphur was determined by fusion with sodium peroxide. Found: S = 11·32 %. Calculated: S = 11·68 %.

1 g. of cystine was recovered from the precipitate which separated during the coupling of the cystine dimethyl ester with the acid bromide. Even allowing for this recovery, however, the yield was poor, as seems to be usual in reactions of this type. Part of the loss may no doubt be referred to the use of mercuric sulphate as a precipitant for cystine and its derivatives [Hopkins, 1921]. From 5 g. of cystine dimethyl ester hydrochloride 0·8 g. of di-hydantoinpropionyl-cystine was isolated.

Opening of the Hydantoin Ring.

The di-hydantoinpropionyl-cystine was dissolved in water and a moderate excess of finely-powdered calcium hydroxide was added. The mixture was boiled gently under a reflux condenser for three-quarters of an hour. At the end of that period the undissolved calcium hydroxide was filtered off and the

filtrate was acidified with dilute sulphuric acid so that the resulting concentration of acid was approximately 1·5 %. On acidification, a certain amount of hydrogen sulphide was evolved showing that a partial decomposition of the cystine portion of the molecule had occurred. This decomposition, however, was less than with sodium or barium hydroxide, the use of which, moreover, would probably result in a greater disruption of the peptide linkage. After standing, the precipitated calcium sulphate was filtered off. No attempt was made to isolate the uramino acid from this solution; it was, however, observed to be precipitated by the mercuric sulphate reagent.

Conversion of the Uramino Acid to the Amino Acid.

Rohde [1918] observed that a uramino acid, treated with nitrous acid, loses half its amino nitrogen. Dr H. D. Dakin, in a private communication, states that by the action of nitrous acid on *β-p*-hydroxyphenyl-*α*-uraminopropionic acid, he has obtained tyrosine. Presumably, an imino acid is first formed and subsequently loses carbon dioxide.

The solution obtained in the previous stage was therefore treated with a dilute solution of potassium nitrite in slight excess of the quantity required for the conversion of the uramino to the amino acid. The solution was allowed to stand several hours, and the dipeptide was then precipitated by mercuric sulphate reagent. The further procedure was as described in the isolation of di-hydantoinpropionyl-cystine. Usually this sufficed, but occasionally it was found advisable to purify the substance further by precipitation of the copper compound of its "reduced" (sulphydryl) form [Hopkins, 1921].

Properties of the Dipeptide.

The dipeptide as thus prepared was a white non-hygroscopic amorphous powder. Like natural glutathione, it was extremely soluble in water, but insoluble in alcohol, ether, and the usual organic solvents. Its aqueous solution was acid to litmus. In neutral solution it gave a violet coloration with triketohydrindene. It gave no coloration with sodium nitroprusside in ammoniacal solution except after reduction with magnesium and hydrochloric acid. On hydrolysis with sulphuric acid, cystine was isolated; with hydrochloric acid, glutaminic acid hydrochloride was isolated.

Amino Nitrogen by the method of Van Slyke. Found: 6·4 %. Calculated for $C_{16}H_{26}O_{10}N_4S_2$: 5·62 %.

It will be recalled that in the case of natural glutathione, Hopkins [1921] obtained a similar high result (6·8 %).

Amino Nitrogen after hydrolysis. After hydrolysis of the substance with 20 % sulphuric acid for six hours, the amino nitrogen was doubled, and, allowing for the somewhat high value to be expected in the presence of cystine, was equal to the total nitrogen. Found: 12·2 %. Calculated: 11·24 %.

Total Nitrogen by Micro-Kjeldahl. Found: N = 11·26 %. Calculated: N = 11·24 %.

Heated in a capillary tube, the substance softened from 165–170° and melted at 187°. A sample of natural glutathione, heated side by side with the synthetic substance, behaved in exactly the same way. Not too much reliance, perhaps, should be placed on the melting point of an amorphous substance, but not only did the two substances melt at precisely the same temperature, but the melt of the synthetic product behaved in the manner characteristic of glutathione, in that there was an evolution of gas, and the liquid ran up the sides of the tube. Further, the melting point was not lowered by mixing the natural and synthetic products.

Optical Activity.

There is thus good agreement between the properties of natural glutathione and of the synthetic dipeptide—and it may be noted that no dissimilarities were observed. The best criterion of complete identity is, of course, the optical activity. From the mode of preparation of the synthetic substance, involving as it does, the closing and opening of a ring by somewhat drastic means, it is obvious that a partial racemisation, at any rate, is to be expected. However, the specific rotation of glutathione being still unknown, it was thought of some interest, at this point, to measure the rotation both of the natural and the synthetic product.

The Specific Rotation of "Oxidised" Glutathione.

I. *In aqueous solution.* 15 cc. of solution in distilled water contained 0·5194 g. of "oxidised" glutathione. The rotation, measured in a 2·0 dm. tube, using the mercury green line, was (mean of six concordant observations) $\alpha = -6{\cdot}82°$. The above solution was diluted to twice its volume with distilled water. Mean of six concordant observations: $\alpha = -3{\cdot}40°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -98{\cdot}3°.$$

II. *In acid solution.* 15 cc. of solution in 10 % hydrochloric acid contained 0·2597 g. of "oxidised" glutathione. Mean of six concordant observations: $\alpha = -3{\cdot}09°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -89{\cdot}2°.$$

The Specific Rotation of the Synthetic Dipeptide.

10 cc. of solution in distilled water contained 0·545 g. of the synthetic dipeptide. The rotation, measured in a 1·0 dm. tube using the mercury green line, gave (mean of twelve concordant observations): $\alpha = -1{\cdot}67°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -30{\cdot}6°.$$

SYNTHESIS II.

Preparation of the acid bromide of glutaminic acid. 10 g. of free glutaminic acid were suspended in 50 cc. of dry toluene. To this suspension were added 3·0 cc. of phosphorus tribromide, *i.e.* a 50 % excess over the quantity theoretic-

ally required to introduce a bromine atom into one carboxyl group. The mixture was boiled gently under a reflux condenser for three to four hours. After cooling it was rapidly filtered at the pump, and the light yellow solid was repeatedly triturated with dry chloroform. It was dried over sulphuric acid and paraffin wax (*in vacuo*). The bromine content of the crude product was estimated in the manner described for the acid bromide of hydantoinpropionic acid. In a typical case, 0·4806 g. gave 0·123 g. of silver bromide, corresponding to a 30 % yield of a mono-acid bromide.

Condensation of the acid bromide with aniline. 4·0 cc. of aniline were suspended in 40 cc. of water and coupled with the acid bromide in the manner previously described for the condensation of hydantoinpropionyl bromide and cystine dimethyl ester (p. 211), except that the solution was not cooled below room temperature. 10 g. of the crude acid bromide were used. After removal of the excess aniline by repeated extraction with ether, the reaction mixture was acidified with hydrochloric acid, and evaporated to dryness *in vacuo*. The residue was extracted several times with absolute alcohol. The combined extracts were decolorised by boiling with a little animal charcoal, and were then evaporated on the water-bath. The crystalline residue was recrystallised from absolute alcohol.

The colourless spear-head crystals were readily soluble in water and alcohol, but insoluble in ether and acetone.

Nitrogen by Micro-Kjeldahl. Found: N = 10·4 %. Calculated for $C_6H_5NH.C_5H_8O_3N.HCl$: N = 10·83 %.

Chlorine by precipitation with silver nitrate. Found: Cl = 13·51 %. Calculated: Cl = 13·73 %.

Calculated for the di-anilide, $C_6H_5NH.C_5H_7O_2N.C_6H_5NH.HCl$:

N = 12·59 %; Cl = 10·64 %.

Oxidation of glutaminyl mono-anilide. A solution in distilled water of the glutaminyl mono-anilide was made faintly alkaline with ammonia, a trace of ferric chloride was added, and the liquid was warmed on the water-bath with excess of hydrogen peroxide. As the hydrogen peroxide was used up, more was added until the reaction had been in progress for one-and-a-quarter hours. At the end of this time it was no longer possible to obtain a coloration with triketohydrindene. The solution was then acidified with hydrochloric acid, and thoroughly extracted with ether, the combined ethereal extracts being dried over sodium sulphate, and evaporated to dryness. There remained a mass of long, fine, colourless needles. These were dissolved in hot water, from which they separated on cooling, a qualitative test on the crude product having shown the presence of nitrogen. As, after recrystallisation, the test for nitrogen was still strongly positive, an estimation by micro-Kjeldahl was carried out. Found: N = 7·34 %. Calculated for $C_6H_5NH.C_4H_5O_3$: N = 7·25 %. The crystals melted sharply at 144–145°. In Beilstein's *Handbuch*, 3rd edition, **2**, p. 413, the melting point of succinyl mono-anilide is given as

148·5°. Later determinations, however, are rather lower than this for it is given (Ergänz. **2**, p. 210) as 144·5–145·5°. Melting point and nitrogen determination, then, agree in identifying the oxidation product as succinyl mono-anilide. There is similar agreement in the reactions. The substance is sparingly soluble in cold water, but readily so in hot water, alcohol or ether. Its aqueous solution is acid to litmus. The addition of silver nitrate to its aqueous solution precipitates the crystalline silver salt. The barium salt is soluble, and cannot be obtained by adding barium salts (or hydroxide) to a solution of the anilide. The calcium salt, on the other hand, is sparingly soluble, and separates in small needles when calcium chloride solution is added to a neutral solution.

Condensation of glutaminyl mono-bromide with cystine dimethyl ester. The coupling was carried out exactly as described in the previous synthesis, care being taken to keep the temperature as low as possible during the reaction. As before, a variable amount of cystine was recovered from the reaction mixture. In one case, from 6 g. of cystine dimethyl ester, 3 g. of cystine were recovered. The dipeptide was isolated through its mercuric sulphate compound as previously described.

Identification of the dipeptide with natural glutathione. The substance thus prepared gave all the qualitative chemical reactions described in Hopkins' original paper [1921] and in this paper for the racemic dipeptide (p. 213). The melting point was identical with that of the natural product, and was not lowered by admixture.

Total Nitrogen by Micro-Kjeldahl. Found: N = 11·35 %. Calculated for $C_{16}H_{26}O_{10}N_4S_2$: N = 11·24 %.

Amino Nitrogen by the method of Van Slyke. Found: 6·4 %. Calculated: 5·62 %. As before, the value obtained for the amino nitrogen is high as is characteristic of the natural product and of cystine compounds in general.

Amino Nitrogen after hydrolysis. The substance was hydrolysed by boiling under reflux condenser for six hours with 20 % sulphuric acid. Found: amino N = 13·26 %. Calculated: amino N = 11·24 %. Found for natural glutathione [Hopkins, 1921]: amino N = 13·3 %.

Sulphur by the method of fusion with sodium peroxide. Found: S = 12·3 %. Calculated: S = 12·80 %.

Specific Rotation. 10 cc. of a solution in distilled water contained 0·5435 g. The rotation, measured in a 1·0 dm. tube, using the mercury green line, gave (mean of twelve concordant observations): $\alpha = -5{\cdot}295°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -97{\cdot}4°.$$

Summary.

I. Glutathione has been synthesised by two methods.

(*a*) Glutaminic acid—hydantoinpropionic acid—hydantoinpropionyl bromide—di-hydantoinpropionyl-cystine—di-uraminoglutaryl-cystine—di-glutaminyl-cystine (glutathione).

(*b*) Glutaminic acid—glutaminyl monobromide—di-glutaminyl-cystine.

II. The product obtained by the first of these methods differed from the natural substance only in its optical activity.

III. The second method yielded a substance completely identical with natural glutathione.

IV. The first method of synthesis substantiates the constitution of glutathione previously advanced by the authors.

V. It is shown that in the acid bromide of glutaminic acid used in the second synthesis, the bromine atom has entered the carboxyl group remote from the amino group. This synthesis, therefore, is also considered to confirm the constitution already deduced.

IV. The specific rotation of glutathione has been measured.

In aqueous solution

$$[\alpha]^{15°}_{\text{Hg. j}} = -98{\cdot}3°.$$

In 10 % hydrochloric acid solution

$$[\alpha]^{15°}_{\text{Hg. j}} = -89{\cdot}2°.$$

VII. The methods described are generally applicable to the synthesis of dipeptides of the type: $\begin{matrix} R \\ R_1 \end{matrix}\!\!>\!CH.NH.CO.CH_2.CH_2.CH(NH_2).COOH.$

The authors have gratefully to acknowledge valuable advice from Dr H. D. Dakin. They also wish to thank Sir F. G. Hopkins, F.R.S., for his constant encouragement.

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XXX. THE EFFECT OF HALOGEN SALTS ON SALIVARY DIGESTION.

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(*Received January 30th, 1925.*)

The experiments described in this paper were suggested by a mishap in an examination. The problem set the students was to find the effect of a given solution on the rate of salivary digestion of starch. The solution was to have been normal potassium fluoride. From experiments previously carried out in the laboratory this was found to delay the appearance of the achromic point considerably, so that instead of its showing in 3–8 minutes it took over 1 hour. For the examination it was necessary to buy a fresh supply of the fluoride, and for reasons of economy it was decided to obtain the sodium salt.

A normal solution of this sodium fluoride was made and used by the fourteen students in the examination. To the surprise of the examiners the vast majority of the class found the fluoride to have no effect on the rate of salivary digestion. This negative result was confirmed by members of the laboratory staff at the time and it was decided to investigate the problem of differences between the action of the sodium and potassium salts. With three separate samples of sodium and potassium fluorides similar results were obtained; namely, no alteration of the time required to reach the achromic point with the sodium salt, and a very delayed action with the potassium salt. It was then thought advisable to see if the salts of the other halogens would show similar differences.

Experimental Method.

Normal solutions of NaCl, KCl, NH_4Cl, $CaCl_2$ and of the corresponding bromides, iodides and fluorides were made. In the case of calcium fluoride which is practically insoluble, a saturated solution was used, but results with this salt are necessarily unreliable owing to the minute amount in solution.

A 0·5 % solution of soluble starch in distilled water was also prepared, and some saliva collected directly into distilled water as described by Cole [1920, 1]. The solutions were warmed separately in a water-bath at 37°, and when at this temperature were mixed in the following proportions: 10 cc. starch; 2 cc. dist. water or salt soln.; 2 cc. saliva. The test-tube was then inverted to mix the contents, returned to the bath, and the time noted on a

stop watch. At intervals of about 0·25 min. a drop of the digestion mixture was taken and added to a drop of weak iodine solution on a white tile. The achromic point was taken as occurring when the drop of digestion mixture and iodine and a drop of distilled water and iodine, placed side by side on the tile, were identical in colour. This was the end point taken in the experiments, and the time was noted on the stop watch.

Thus these experiments were carried out on saliva with its natural salt content, and not on ptyalin itself. The need for stressing this point was shown by Cole [1903]. It was thought better to use saliva as such, since it is impossible to obtain an absolutely pure ferment, and also because salivary digestion is normally carried out by saliva and not by a dialysed extract.

Experimental Results.

Chlorides.

Standard with water ...	(1)	8·0 mins.
	(2)	8·0 ,,
NaCl	(1)	3·75 ,,
	(2)	3·55 ,,
KCl	(1)	3·75 ,,
	(2)	3·75 ,,
NH_4Cl	(1)	4·80 ,,
	(2)	5·0 ,,
$CaCl_2$	(1)	3·75 ,,
	(2)	3·75 ,,

Hence all these chlorides, as previously shown by many workers, accelerated diastatic action.

Fluorides.

Standard with water ...	(1)	8·0 mins.
	(2)	8·2 ,,
NaF...	(1)	8·5 ,,
	(2)	8·3 ,,
KF	(1)	120·0 ,,
	(2)	125·0 ,,
NH_4F	(1)	162·0 ,,
	(2)	180·0 ,,

This ammonium salt mixture seemed to be very acid, therefore ammonium hydroxide was added till it was markedly alkaline. The figures then were:

$NH_4F + NH_4OH$ ...	(1)	56·0 mins.
	(2)	62·0 ,,
CaF_2	(1)	8·0 ,,
	(2)	8·3 ,,

Here there is a marked difference in the various fluorides, sodium fluoride being inert, whilst potassium and ammonium fluorides exert a marked slowing effect. As stated before, owing to its insolubility, the experiments with calcium fluoride are not comparable and are inconclusive.

Bromides.

Standard with water	(1)	8·0 mins.
	(2)	8·0 ,,
NaBr, KBr, NH_4Br, $CaBr_2$ ...		8·0–8·2 mins.

Hence the bromides were without any action whatever on the rate of salivary digestion.

Iodides.

Standard with water ...	(1)	12·0	mins.
	(2)	12·0	„
NaI	(1)	12·0	„
	(2)	12·0	„
KI	(1)	12·25	„
	(2)	12·75	„
NH_4I	(1)	19·25	„
	(2)	20·0	„
CaI_2	(1)	44·0	„
	(2)	44·75	„

Here, as with the fluorides, there is a marked difference, the sodium and potassium salts being inert as regards effect on the rate of salivary digestion, whilst the ammonium and calcium salts delay it to a marked extent.

It was then thought that the differences might be due to variations in the acidity of the salt solutions since this factor is so important in physiological reactions, and the salt solutions were known to show large differences in their behaviour to indicators.

Accordingly the hydrogen ion concentration of these salts was estimated by Cole's comparator method [1920, 2] using phenol red as the indicator. The results are given in the following table:

Effect of salts on rate of Salivary Digestion.

Hastened action		Unaltered action		Delayed action	
NaCl	p_H 7·0	NaF	p_H 7·6	NH_4I ...	p_H 5·6
KCl	6·6	*(CaF_2) ...	7·2	CaI_2	8·0
NH_4Cl ...	5·6	NaI	8·0	KF	7·8
$CaCl_2$	7·2	KI	7·0	NH_4F ...	6·6
		NaBr ...	6·8	$NH_4F + NH_4OH$	8·0
		KBr	6·8		
		NH_4Br ...	5·8		
		$CaBr_2$ ...	8·0		

* Very insoluble, and therefore not really comparable.

From this table it appears that the chlorine ion exerts a definite accelerating action on salivary digestion rate, that the bromine ion is inert, and that the metallic ion is relatively unimportant in this connection.

With the fluorides and iodides there are marked differences, some of the salts delaying digestion, whilst others are inert. This difference does not seem to be due either to the metallic ion, or to the hydrogen ion concentration of the salt solution, but to be a definite effect of the salt as a whole, independent of the hydrogen ion concentration or the position of the ions in the periodic system.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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XXXI. THE INFLUENCE OF DIFFERENT SUBSTANCES ON THE DIASTATIC ACTIVITY OF SALIVA.

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From the Department of Physiology, University of Birmingham.

(*Received February 20th, 1925.*)

This research was undertaken to determine the action of sweet substances on the diastatic activity of saliva, both as regards the secretion of ptyalin, and its activity when produced.

Recent work, which has a bearing on the subject, is as follows.

Iljin [1922] has found that salts accelerate the production of diastatic enzymes, probably by hastening the transition from the inactive zymogen to the active zymin. Rockwood [1918] showed that the ammonium salts of inorganic acids increased the activity of ptyalin, as did also those of the stronger organic acids. Groll [1918] showed that anions exert a greater influence than cations. The halogen ions increase and those of the sulphates and nitrates inhibit the activity of ptyalin. Ambard [1923] has found that starch adsorbs a definite amount of salivary amylase. Neilson and Lewis [1908] determined the effect of diet on the amylolytic power of saliva. They found that after feeding for some days on a carbohydrate diet the amylolytic power is increased, whilst it is decreased on a protein diet. Sanctis-Monaldi [1920] found that the secretion of parotid saliva was increased by various stimuli, such as bread, meat and acid, but its amylolytic power remained unchanged.

Method. Various substances were put into the mouth and rolled between the tongue and palate, and the activity of the saliva so secreted was compared with that of the saliva previously secreted in the empty mouth. The saliva was as a rule collected between 10 and 11 a.m., breakfast having been taken about 8 or 8.30. In each experiment the mouth was rinsed out three times with tap water and three drinks of water were taken; the saliva then collected was called *N*. After its collection the same procedure was gone through; the substance to be tested was put into the mouth and the saliva so produced called *S*. In each case the saliva was collected into a dry dish. 1 cc. was transferred to a 100 cc. flask, which was then filled to the mark with distilled water. The liquid was then filtered. Throughout the experiment the flasks, dishes and test-tubes were boiled before use.

The estimation of the diastatic activity was carried out by a modification of Wohlgemuth's method.

In a series of twelve test-tubes the diluted filtered saliva was added to a 0·9 % solution of sodium chloride according to the following table, each test-tube thus containing 1 cc. of liquid.

Test-tube	cc. diluted saliva	cc. 0·9 % NaCl
1	0·35	0·65
2	0·33	0·67
3	0·31	0·69
4	0·29	0·71
5	0·27	0·73
6	0·25	0·75
7	0·23	0·77
8	0·21	0·79
9	0·19	0·81
10	0·17	0·83
11	0·15	0·85
12	0·13	0·87

To each tube was then added 2 cc. of 0·1 % solution of starch. Soluble starch was used; the solution was free from chloride and neutral to litmus.

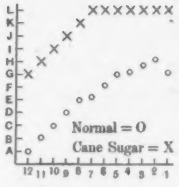
The set of tubes was then put into an incubator for 30 minutes. The temperature of incubation was as a rule 38°, but in various experiments there were slight variations above and below this.

After incubation the tubes were cooled by transference for 3 minutes to water at the temperature of the laboratory. To each tube was then added one drop of a $N/50$ solution of iodine.

Instead of taking the achromic point as a measure of diastatic activity it was found more convenient to make a series of standard colours ranging from blue through purple, red-purple, red-brown to yellow, and by comparison of the experimental colours with those of a standard, to plot a graph for the diastatic activity. The preparation of the standard colours was difficult, as it was not easy to match the exact tint, and when this was done, often after a few days it was found that a precipitate had appeared, or that the colour had faded. Mixtures of the following solutions were found to be most satisfactory: methylene blue, Uffelmann's reagent, magenta, carbol fuchsin and Bismarck brown. The exact strength and proportion of each solution cannot be given, as in each case it was a matter of repeated trial, adding first a little of one and then a little of another until a match was obtained to the colours produced by iodine on partially digested starch at different stages.

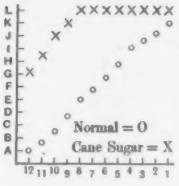
The colours were as follows:

A = Blue ≡ no digestion.
B = Blue with a tinge of purple.
C = Purple.
D = Red-purple.
E = Magenta.
F = Red.
G = Pale red-brown.
H = Pale red-brown, but more brown and less red than G.
I = Pale brown with a tinge of red.
J = Paler brown with a tinge of red.
K = Yellow-brown.
L = Yellow ≡ complete digestion.



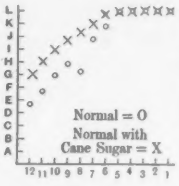
Subject H. W.

Graph 1.
Cane Sugar



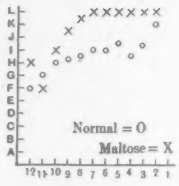
I. J.

Graph 2.
Cane Sugar



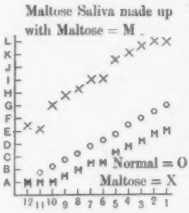
H. W.

Graph 3.
Normal Saliva made up with 10 per cent. Cane Sugar



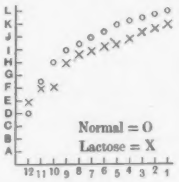
H. W.

Graph 4.
Maltose



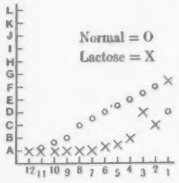
W. M.

Graph 5.
Maltose



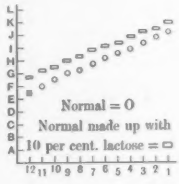
H. W.

Graph 6.
Lactose



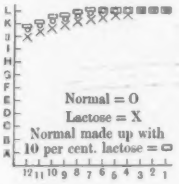
D. P.

Graph 7.
Lactose



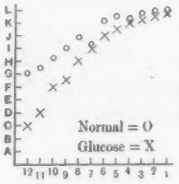
H. W.

Graph 8.
Lactose



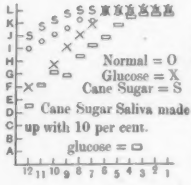
H. W.

Graph 9.
Lactose



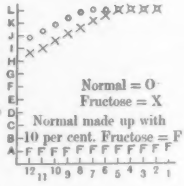
H. W.

Graph 10.
Glucose



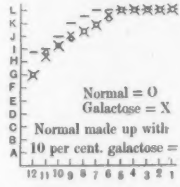
H. W.

Graph 11.
Glucose, Cane Sugar



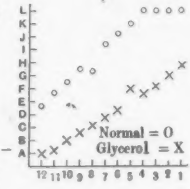
H. W.

Graph 12.
Fructose



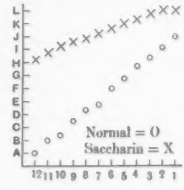
H. W.

Graph 13.
Galactose



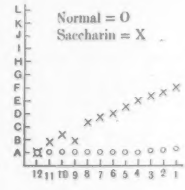
H. W.

Graph 14.
Glycerol



H. W.

Graph 15.
Saccharin



E. W. C.

Graph 16.
Saccharin

The numbers on the base lines are those of the series of tubes.

Since the experiments were comparative no determinations of the p_H were made. It is assumed that in each experiment it was the same for the normal and stimulated saliva, as the substances used were as far as possible pure. In any case the dilution was so great that a slight variation in the p_H would make very little difference.

In graph 1 it will be seen that the starch in the tube containing the smallest amount of normal saliva has undergone no digestion, while the corresponding tube of saliva after cane sugar shows considerable digestion. In each series the amount of digestion increases with increasing quantities of saliva, but in the *S* set digestion is complete in tube 7 and the following tubes, and presumably with a larger quantity of starch the curve would continue its upward direction, while in the *N* set, tube 2 with greatest digestion is only equivalent to tube 11 of the *S* set. A similar result is seen in graph 2.

From these graphs it is seen that cane sugar in the mouth has a very definite accelerator action on the saliva produced.

In order to determine whether this increase in activity was due to an increase in the quantity of ptyalin, or to an activating effect of the sugar upon it when produced, some normal saliva was collected in the usual way; of this 1 cc. was made up to 100 cc. with distilled water, and 1 cc. was made up to 100 cc. with 10 % cane sugar solution. The result is shown in graph 3.

From this it is seen that the presence of cane sugar increases the activity of ptyalin.

Maltose (graph 4) was found also to accelerate the action of saliva. In order to determine whether this was due to activation of the ptyalin, 1 cc. of the maltose saliva was made up to 100 cc. with 10 % maltose solution. The effect, as seen in graph 5, was to depress the action, and therefore the stimulating effect of maltose in the mouth must be due to a greater secretion of ptyalin.

The results of lactose in the mouth are shown in graphs 6 and 7; it appears to have an inhibitory action on saliva. In the next experiment 1 cc. of normal saliva was made up to 100 cc. with 10 % lactose solution, and the activity of this solution was compared with that of the normal saliva made up with saline in the usual way. The result is recorded in graph 8, from which it appears that the presence of lactose in solution enhances the activity of ptyalin. The result of a further confirmatory experiment is shown in graph 9.

Glucose in the mouth also has an inhibitory effect (graph 10).

In graph 11 the result of a comparison between normal, cane sugar, and glucose saliva is shown. The inhibitory effect of glucose was also determined by making up 1 cc. of the cane sugar saliva to 100 cc. with 10 % glucose solution. The graph shows the very marked inhibitory action of glucose.

Fructose in the mouth has a depressing effect, and that this is due mainly, if not entirely, to an inhibition of the ptyalin is seen from graph 12, which shows the result of making up 1 cc. of normal saliva with 10 % fructose solution, in which case there is practically no digestion at all.

Galactose seems to have very little effect, though its presence in solution slightly increases the activity of ptyalin (graph 13).

Glycerol (graph 14) in the mouth has a retarding influence. Saccharin, of which the sodium salt was used, has an activating effect (graphs 15 and 16).

Summary.

From these experiments it is apparent that when various substances are taken into the mouth two effects have to be considered:

(1) an increase or decrease in the quantity, or possibly the quality, of the ptyalin secreted,

(2) a stimulating or depressing action on the enzyme produced by the substance being tested.

Of the substances examined, cane sugar and saccharin in the mouth appear to have the most marked effect in producing a very active saliva. In the case of cane sugar this is largely, if not entirely, due to its stimulating action on the ptyalin.

The behaviour of maltose and lactose is interesting; while maltose increases the quantity of ptyalin produced, its presence depresses the activity of the enzyme. Lactose, on the other hand, tends to inhibit the production of ptyalin, but enhances its activity.

Of the monosaccharides glucose and fructose both lower the activity of saliva, and in each case it is largely due to the depressing action of the sugar on ptyalin.

Further investigations on the same lines are proposed.

My best thanks are due to Professor Carlier (at whose suggestion the work was begun) for much helpful advice and criticism.

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XXXII. A COMPARISON OF DRIED AND EVAPORATED MILKS BY A DIETETIC METHOD.

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(*Received February 12th, 1925.*)

At the present time there are a great number of dried and evaporated milks on the market, and many of them appear to have been used successfully in infant feeding. In fact, some makes are regarded as superior to fresh cow's milk, in that there is no possibility of the milks turning sour. Dried milks are made by one of two methods, either the "roller" or the "spraying" process being used. There seems to be no conclusive evidence as to which method supplies the best results, except with regard to vitamin C, when less destruction is claimed with the "roller" process [Jephcott and Bacharach, 1921; Hart, Steenbock and Ellis, 1921]. Experimental work on this subject has been done by various observers, a survey of the literature being given in the report of the Medical Research Council [1924].

The experiments to be described in this paper were started in the hope of obtaining information as to the vitamin B content of dried and evaporated milks. It had been found previously [Hartwell, 1921] that an excess of protein in the lactating rat's diet resulted in the offspring's suffering from spasms, etc., and that the young rarely survived. Later [Hartwell, 1922] it was shown that the addition of 100 cc. of whole cow's milk to the mother's excess protein diet entirely prevented the abnormal symptoms in the sucklings. Therefore it seemed possible that this fact might be utilised as a basis for comparison of different kinds of dried and evaporated milks.

Experimental.

1. *General Principle of Method.*

A diet was chosen which had been found to cause death of the offspring, while the mother remained fit and well. To this basal diet were added different quantities of fresh cow's milk in order to find the least amount necessary to protect the young from spasms. Dried or evaporated milks were then substituted in turn for the fresh cow's milk and the results compared.

2. *Diets.*

The basal diet consisted of bread and food casein [Hartwell, 1922] fed in the proportion of 15 g. bread to 6 g. food casein. To this basal diet were added successively 75, 50, 33, 24, 18 and 12 cc. of fresh cow's milk, water being added when necessary to make the mixture into a paste.

(*a*) *For the dried milks* the following proportions were used:

Dried milk,	full cream,	12 g.	to	100	cc.	water.
,,	,,	half ,,	10·5 g.	,,	100	,,	,,
,,	,,	skim,	9 g.	,,	100	,,	,,

These amounts were used in place of 100 cc. of cow's milk. They were chosen in accordance with the directions on the tin, the object being to use the same amount of powder as would be taken by the average person making up the milk from the given directions. Therefore different people were asked to measure out the amount stated on the tin and these amounts were weighed. For example, take x tablespoonfuls of dried milk to y pints of water. Twenty people were asked to measure x tablespoonfuls; these different quantities were then weighed and the average obtained. This amount was used with y pints (converted to cc.) of water. It is interesting that the figure obtained in this way is near the average of 12·4 % given for total solids in milk [Plimmer, 1921], but, of course, using this proportion does not allow for any water in the dried milk.

(*b*) *For evaporated milks*, the dilution used was that given on the tin, and this diluted milk was substituted for cow's milk. The specific gravity of the liquid was approximately that of fresh cow's milk.

The control mothers were fed on bread and milk, 15 g. bread to 50 cc. cow's milk (or its equivalent).

3. *Method.*

At least three mother rats were given each diet, and they received food *ad lib.* The mother and young were weighed daily. The babies were watched carefully for any abnormal symptoms. The mother rats had a good mixed diet during gestation and only litters of good weight at birth were used in these experiments. The special diet was given as soon as possible after the birth of the litter. In all, 171 mother rats and litters with 6 young to a litter were used in these experiments.

Results.

The results are given in the Table, p. 228. In every case the condition of the young became worse as the mothers received smaller proportions of milk (Fig. 1, cow's milk is given as illustration). It is obvious that fresh and dried milks much excel the evaporated milks in vitamin B content. For example, it is significant that 75 cc. of diluted evaporated milk are insufficient to prevent spasms in the young, while 33 cc. of cow's milk or the equivalent amount of some dried milks are almost sufficient, no spasms and only slight

Proportions of Milk added to Basal Diet of Mother Rat.

Sample	Control	I. 75 cc.	II. 50 cc.	III. 33 cc.	IV. 24 cc.	V. 18 cc.	VI. 12 cc.	Remarks
Cow's	50 cc. milk to 15 g. bread	Normal condition. Growth curve depressed	Same as I	Growth about the same as in I, but animals not quite normal, slight incoordination shown. 1 out of 18 died	Growth more depressed than in I, II and III. All 3 litters showed spasms and 1 litter had screaming fits	Growth similar to IV, but curve flattened from 15th day, some loss of weight. All 3 litters had spasms and screaming fits. 1 out of 18 died	Condition of young very bad from 12th day. All 3 litters showed typical symptoms and 2 whole litters died, *i.e.* 12 out of 18	—
Dried.								
A ("roller")	Rate of growth of sucklings equal to that obtained with cow's milk	—	Same as with cow's milk	Growth not quite so good as in II. Slight spasms shown. 1 out of 18 died	Growth about same as III. All 3 litters showed incoordination. 1 litter had spasms and screaming fits. None died	Growth similar to III. 2 litters spasms and 1 litter screaming fits. 1 out of 18 died	Growth slower but 2 litters successfully weaned, although all 3 litters abnormal. Two young died	Slightly better than cow's milk
B ("roller")	Do.	—	Same as with cow's milk, but slight incoordination in 1 litter. 1 baby died	Same as II	Growth about the same as II, but all 3 litters had spasms and screaming fits. 1 baby died	Growth and condition very poor, 2 whole litters and 2 other young died, *i.e.* 14 out of 18 died	Condition very bad from 12th day. 1 whole litter died, and 5 from each of the other litters, *i.e.* 16 died	Slightly worse than cow's milk
C ("roller")	Do.	—	Same as II B	Growth depressed, all 3 litters spasms; 1 litter did not gain weight after the 14th day	Similar to III	Condition of young poor. 2 whole litters died, the other showed no gain after the 15th day	Condition very bad but 7 survived	Slightly worse than cow's milk and similar to sample B
D 1 ("roller") (full cream)	Do.	—	Growth depressed, 2 litters had spasms, and in addition had screaming fits, *i.e.* only 1 litter was normal	Growth same as II. All 3 litters had spasms, and 1 litter screaming fits	Growth depressed and curves very flattened from 14th day. All 3 litters had bad spasms and 2 had screaming fits. 1 out of 18 died	Condition very bad, all 3 litters died	—	Worse than cow's milk
D 2 ("roller") (half cream)	Not quite so good as D full cream	—	Very poor growth; all 3 litters had spasms and screaming fits. 5 died	Same as II, but only 1 died	Same as III, only 1 died	—	—	Not quite so good as D full cream

E ("spray") (full cream)	Normal	—	All 3 litters had spasms and 3 young died	—	—	All 3 litters had spasms and 1 whole litter died	—	About the same as D full cream
F 1 (full cream) ("spray")	Rate of growth of sucklings equal to that obtained with cow's milk	—	Growth quite good, condition normal	Same as II; no spasms	—	All 3 litters had spasms and screaming fits. Growth curve flattened from 14th day. None died	All litters had spasms. 1 whole litter and 2 other babies died, *i.e.* 8 died	Slightly better than cow's milk; equal to A
F 2 (skim) ("spray")	Almost maximal growth	—	Growth good, but not up to standard. No spasms	Growth good. 2 litters had spasms and all 3 showed incoordination	—	Growth curve very depressed, all 3 litters spasms. 1 whole litter died	—	Not so good as F 1
Evaporated.								
G	Rate of growth a little slower than with cow's milk; young rats had thin coats and were very limp to the touch	Growth curve depressed. All 3 litters exhibited incoordination and 2 had spasms. 1 baby died	Same as I, but condition of young just worse. All 3 litters had spasms and screaming fits. 1 died	Very slow growth of young. All 3 litters showed spasms and screaming fits. 1 died	Same as III, but 2 died	Condition worse than IV, 1 whole litter and 2 other young died	—	Not quite so good as D 1, distinctly poorer than cow's milk
H	Similar to G	—	Growth very poor indeed, 2 litters ceased to gain weight from the 14th day. All 3 litters had spasms and screaming fits. 2 died	Condition similar to 2, but worse. 9 died	Similar to III. 8 died	Condition worse than IV. 12 died	—	Not quite so good as G
I	Similar to G and H	—	Growth curve depressed. 1 litter had bad spasms. 5 died	—	All 3 litters had bad spasms and screaming fits. 1 whole litter died and 3 other babies	—	—	—
J*	—	Growth fairly good, but 1 litter had spasms and 1 litter screaming fits	—	—	—	—	—	—

* It was impossible to obtain any more of sample J.

incoordination being noted. A comparison of some of the samples is shown in Fig. 2.

The control experiments (where the mother rats were fed on bread and milk alone, *i.e.* no extra protein) also are interesting, in that they support

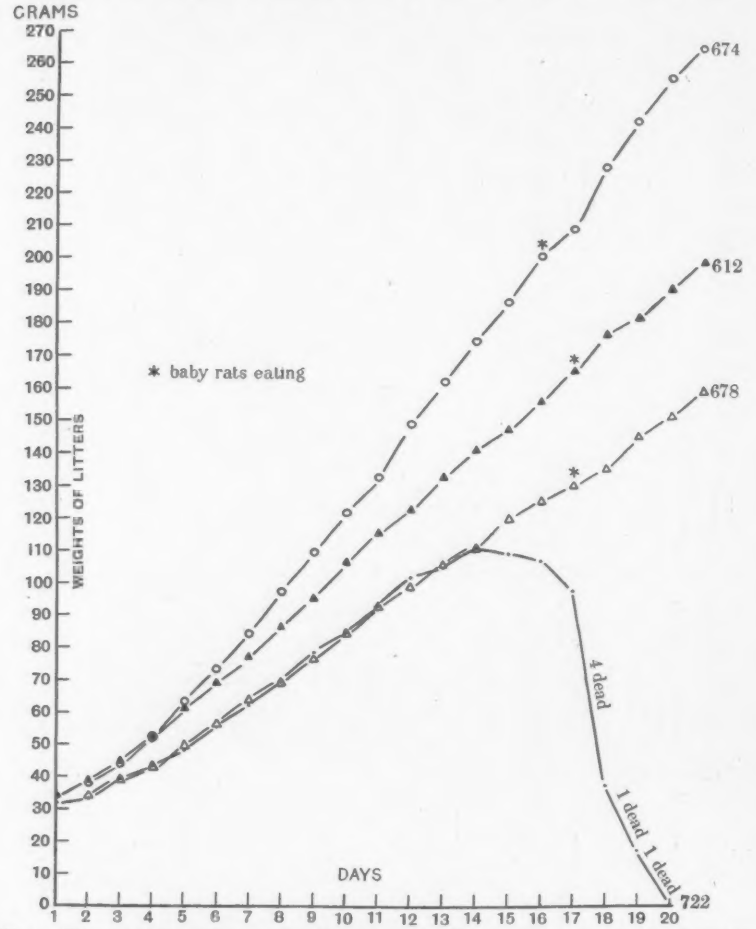

Fig. 1. Effect of adding different proportions of cow's milk to a basal diet of "excess" protein.

Mothers' diets.

○——○ 674. 15 g. bread, 50 cc. cow's milk. Control.
▲——▲ 612. 15 „ „ 6 g. food casein, 50 cc. cow's milk.
△——△ 678. 15 „ „ 6 „ „ „ 24 „ „ „
•——• 722. 15 „ „ 6 „ „ „ 12 „ „ „

the general view that evaporated milks are not so good as the dried varieties. The young belonging to the mothers fed with dried milk and bread were

normal, and appeared equal in all respects to the young whose mothers had bread and fresh cow's milk. The young whose mothers received evaporated

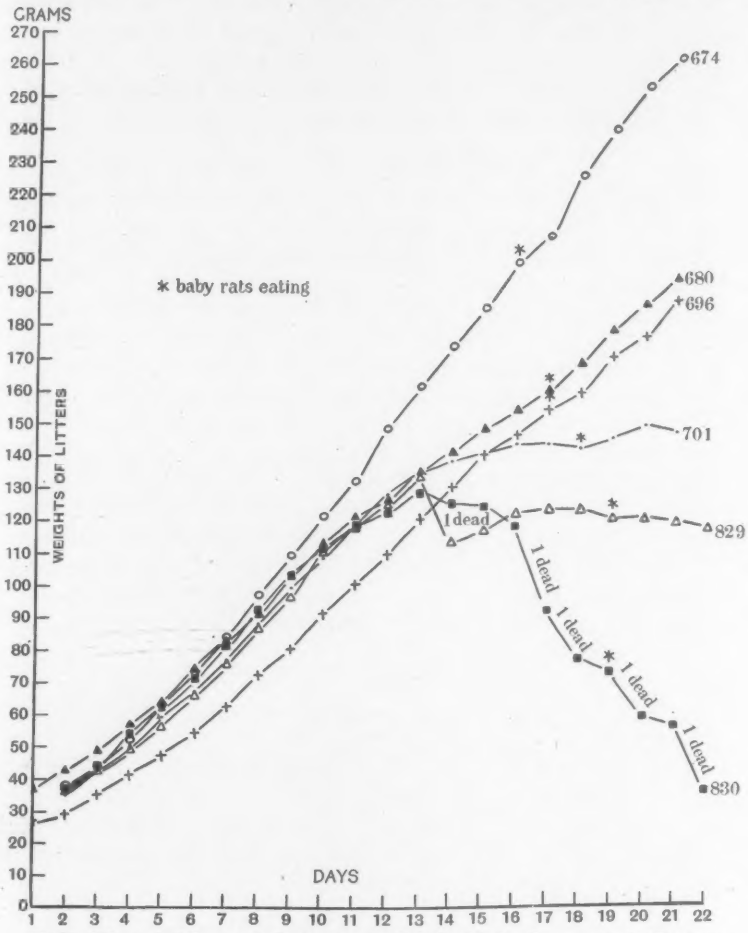

Fig. 2. Effect of adding equivalent amounts of cow's, dried and evaporated milks to a basal diet of "excess" protein.

Mothers' diets.

○——○	674.	Bread and cow's milk. Control.
▲——▲	680.	Basal diet + cow's milk.
+——+	696.	„ „ + dried milk; sample A.
•——•	701.	„ „ + „ milk; „ D 1.
△——△	829.	„ „ + evaporated milk; sample G.
■——■	830.	„ „ + „ milk; „ H.

milks were by no means up to standard. Their weights certainly were quite good, though slightly below normal, but their coats were poor and the baby

rats were limp to the feel. In other words they were just not fit. Confirmatory results were obtained in a series of experiments, to be published shortly, on the growth of weanling rats on dried, evaporated and fresh cow's milk. Summing up one can say that samples A and F1 (dried milks) are just better than cow's milk, and samples B and C (also dried milks) just less good, while samples G, H, I and J (evaporated milks) are distinctly inferior as compared with cow's milk in regard to vitamin B content.

Comments and Conclusions.

The evidence is quite conclusive that evaporated milks are poorer in vitamin B content than is fresh cow's milk (heated to 70°). With dried milks on the other hand, there is no definite evidence as to which method ("spray" or "roller" process) produces the better results, since sample F1 ("spray") is quite as good as sample A ("roller"), while sample E ("spray") is as poor as sample D1 ("roller"). It is quite possible that some individual differences in manufacture are responsible for these variations.

As pointed out by Heineman [1919] dried milks prepared by the "spray" process are more easily soluble in water than are those prepared by the "roller" process. Also the former are easily clotted by rennet, *e.g.* sample E clots in approximately the same time as cow's milk, while a true junket is not obtainable from samples A and B. It may be that the manufacturing method most suitable for preserving the physical properties of milk is one which results in a greater destruction of vitamin B, and that a method safeguarding vitamin B results in an alteration in the physical condition of the proteins, etc., of the milk. It would be interesting to know if vitamin A suffers any similar destruction.

Summary.

1. A dietetic method is described for the comparison of cow's, dried and evaporated milks as regards vitamin B content.

2. The vitamin B content of evaporated milks is lower than that of dried milks and of fresh milk.

3. Dried milks vary in their vitamin B content, which may be slightly greater or slightly less than that of fresh milk. This variation does not run parallel with the method of manufacture.

4. Dried skim and half-cream milks are not quite so good as the same makes in full-cream.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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XXXIII. MERCURIALIS.

I. THE DEVELOPMENT OF A BLUE PIGMENT ON DRYING.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department of University College, London.

(*Received February 25th, 1925.*)

It has long been known that the green shoots of *Mercurialis perennis* and *M. annua* acquire a bluish hue on drying, a fact that is perhaps not as widely known to botanists as might be expected. The only reference to the nature of the blue pigment which we have been able to trace is one by Molisch [1893], who, in the course of a systematic search for indigo-producing plants examined *M. perennis* and dismissed this plant with the remark that the pigment is not indigo, since it is turned red by the addition of acid.

With regard to the mechanism of the production of the blue colour the possibility of enzyme action at once suggests itself since, in a great many cases, the post-mortem coloration of vegetable tissue has been shown to be due to the presence either of an oxidase acting upon some constituent of the substrate or of a hydrolytic enzyme acting upon a glucoside, as has been shown by Bridel and Braecke [1922, Bridel, 1923] for *Melampyrum*, *Monotropa* and other plants.

In the present instance of *Mercurialis*, however, it would appear from our observations that enzyme action of any kind is highly improbable. The possibility of oxidase action is excluded since this plant does not contain a direct acting oxidase but only a peroxidase which is incapable of exerting its activity until after the addition of a peroxide. The possibility of other enzyme action is unlikely in view of the fact that the blue pigment can be produced by drying the tissues after such treatment as immersion in boiling water or suspension in steam; further the pigment may be produced by leaving the tissues to soak for some time in absolute alcohol, a reagent which is not generally regarded as favourable for enzyme action. Our observations lead us to conclude that desiccation is the prime factor concerned in the production of this blue pigment.

The Preparation of the Blue Pigment by Desiccation.

(*a*) *By the use of absolute alcohol.* This reagent, as is well known, rapidly removes water from fresh tissues and also quickly expels the air contained within the intercellular space system of the plant, a point of some importance

in view of the formation of another blue pigment by oxidation. The use of absolute alcohol enables one either to extract the blue pigment or, by slightly modifying the conditions, to bring about its deposition within the tissues. If the former be desired it is necessary to use the colourless rhizomes in order to avoid contamination with chlorophyll. The freshly-gathered rhizomes are washed free from adhering soil, dried with a cloth, rapidly cut up into short lengths and dropped into absolute alcohol contained in a flask; the latter is then gently warmed over a water-bath to a temperature not exceeding 40°. The resulting deep blue solution is filtered into a distillation flask and evaporated to small bulk under reduced pressure in a stream of nitrogen. The residual liquid consists of a violet coloured aqueous solution in which is suspended a flocculent greyish white substance, which on filtration and exposure to air darkens. This material contains phosphorus, and from its solubilities and general behaviour, appears to be of a lecithin-like nature, but it has not as yet been fully investigated. The aqueous filtrate on evaporation to dryness yields a dark blue material which sets to a brittle varnish.

If it is desired to produce the pigment in the tissues and to leave it there deposited with a view to studying its anatomical distribution, advantage may be taken of the circumstance that the pigment is insoluble in absolute alcohol and only comes out into solution if the alcohol is allowed to become diluted by the moisture withdrawn from the tissues. To study the distribution in green leaves or shoots it is therefore necessary to immerse them in absolute alcohol and to replace the alcohol frequently, as it takes up moisture from the tissues; in these circumstances only the chlorophyll goes into solution and the alcohol is accordingly coloured green and not blue. If such an experiment is carried out successfully the tissues gradually lose all their chlorophyll and assume an intense azure blue colour. The blue pigment can be removed from such tissues by immersion in water.

(*b*) *By drying.* In the early phases of this investigation it was observed that on drying the material developed the blue pigment very irregularly. In order to discover the best method, green shoots and leaves and colourless rhizomes were dried under a great variety of conditions, as a result of which it was found that the best yield of blue is obtained by drying as slowly as possible, in a dark cool place. Under these conditions of slow drying there is less tendency for the production of the unstable and readily oxidisable blue compound to be described later; the choice of a dark place is to be recommended more particularly in the case of green material, since there is less danger of contamination with possible degradation products of chlorophyll.

To extract the pigment from the tissues, the dried material is ground in a mill and a quantity of the resulting powder is stirred into cold water until the powder is thoroughly wet. The pigment rapidly diffuses out and the extraction is complete in half an hour, if the paste is kept well stirred. The material is then placed in a linen bag and expressed in a tincture press. The greenish-blue liquid thus obtained may be used for extracting a fresh quantity of the powder

and by so doing a strong solution results. This is centrifuged and, if necessary, filtered.

Reference has already been made to the production, by oxidation, of a second blue compound which on further oxidation gives a yellow one. There is good reason to infer, as will be seen later, that both these blue compounds are produced from the same mother substance and that if the chromogen is converted into the transient blue compound it cannot form the permanent blue substance.

It is the production of the yellow substance, in varying amount, which is responsible for the fact that in a length of dried rhizome a portion may be yellow while the rest is peacock blue. The yellow compound being likewise soluble in water is extracted together with the permanent blue and is thus responsible for the peacock green colour of the extract. To remove this impurity, the centrifuged solution is saturated with ammonium sulphate, whereby the blue compound is precipitated; the filtrate, which contains the yellow compound together with a small additional quantity of the blue, is bright green in colour; on prolonged standing a little more of the blue compound is thrown out. The precipitated pigment is filtered off, washed with saturated ammonium sulphate solution and dried at 37°. The chemical properties of this substance are still under investigation.

Distribution of the Blue Pigment.

All parts of the dried plant, aerial shoots and subterranean rhizomes and roots, may show a peacock blue coloration, which appears in the last phases of desiccation, the colour being densest in the younger parts. Thus the apex of the shoot contains more pigment than the basal region, young leaves contain more than old, young white rhizomes are much richer than the older brown. In axial structures the pigment is restricted to the cortical parenchyma and in the leaves it is distributed more or less evenly throughout the parenchyma of the mesophyll.

Summary.

1. There exists in *Mercurialis* a chromogen which gives rise to a blue pigment on drying.

2. The production of the blue colour is not due to enzyme action.

3. Methods are described for the isolation of the pigment.

4. The yield of the pigment indicates that the chromogen is most abundant in the youngest and vigorously growing parts.

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XXXIV. MERCURIALIS.

II. THE OCCURRENCE OF A CHROMOGEN SHOWING A REMARKABLE AVIDITY FOR FREE OXYGEN.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department of University College, London.

(*Received February 25th, 1925.*)

With the object of extracting from *Mercurialis* the precursor of the permanent blue compound whose isolation is described in the previous paper [Haas and Hill, 1925], fresh tissues were closely packed into a glass cylinder provided with a glass stopper; the tissues were then completely covered with 10 % alcohol, the cylinder being filled to the top and the stopper inserted. On removing the stopper after 48 hours and withdrawing the tissues, the aqueous extract was found to be colourless, but after a few minutes the superficial layer assumed a blue colour which slowly disappeared on further exposure to air. These facts indicate that *Mercurialis* contains a chromogen which by oxidation yields a transient blue compound distinct from the permanent blue compound mentioned above. In view of the sensitivity of this chromogen to atmospheric oxidation, the following method of preparation was adopted.

Exp. 1. Cleaned white rhizomes or apical regions of young aerial shoots are cut into short lengths, the severed portions being allowed to fall into a wide-mouthed glass bottle containing just enough cold freshly-boiled water to cover the fragments of tissue used. Roughly 100 g. of rhizome cut into quarter-inch lengths require about 100 cc. of liquid, whilst the same weight of aerial shoot will need about 175 cc. of water. About 2·5 cc. of chloroform for each 100 cc. of water are then poured in and a rubber cork, fitted with inlet and outlet tubes, is rapidly placed in position, and a rapid stream of nitrogen is passed through in order to remove the air. After a few minutes the nitrogen stream is moderated and the bottle is placed in a thermostat at 45° for one hour.

If the experiment is carefully conducted the liquid in the bottle will be colourless, but on removal and filtration a striking change takes place; the extract rapidly alters in colour to a deep azure blue which may be preserved for some time if the solution be kept in an atmosphere of nitrogen. If, however, the extract be fully exposed to air, the blue increases to a maximum and then changes through green to yellow, the latter being permanent; the green phase is due to a mixture of the yellow with the blue. The depth of colour developed

varies with the strength of the solution; thus from vigorous young shoots the blue pigment may be so deep as to appear almost black and this, on complete oxidation, will give a brown colour. The residual tissues remaining after extraction are no longer able to form the permanent blue on drying.

Another way of demonstrating the colour changes resulting from atmospheric oxidation is to fill a glass cylinder with the blue solution and to leave it open to the air. In time the surface layer will change to yellow, so that a sharp line of demarcation is formed between the yellow and the blue. This line moves slowly downwards as the oxidation of the blue compound proceeds. The sharp separation between the yellow column above and the blue column below is easily broken, wherefore the greatest care must be taken to avoid shaking and the formation of convection currents. This also serves to demonstrate the rate of solution and diffusion of oxygen into the solution and it is possible that this pigment may find application in the investigation of gaseous diffusion, not only in liquids but also in gels.

A similar cycle of changes in colour can also be produced in the tissues by cutting, bruising or otherwise injuring them. Thus if a fresh piece of white rhizome is cut across with a sharp knife, the cortical layer will in a short time assume a blue colour which gradually fades away. Or again, if a small quantity of rhizome is crushed in a white porcelain dish the material quickly assumes a blue colour which changes to dull green and finally to yellow. If the tissue has been well crushed and frequently turned over to ensure thorough exposure to air the pulp will no longer give rise to the permanent blue on desiccation. Similarly it is found that exposure to chloroform vapour induces in a colourless rhizome the formation of the transient blue, the oxygen in this case being presumably supplied by the air contained in the intercellular space system of the tissues. Here again it is found that a portion of the tissue which has been exposed to chloroform vapour for a sufficient length of time to ensure the maximum formation of the transient blue and its subsequent oxidation, will no longer yield the permanent blue on drying. This may be conclusively shown by passing a long rhizome through a cork into a test-tube containing a little chloroform, in such a way that part of the rhizome is within the tube and the part on the other side of the cork is exposed to the air. The enclosed portion will soon turn blue and next day it will be yellowish white, whilst the part exposed to the air will be blue, owing to the formation of the permanent blue.

Exp. 2. That the changes here described are due solely to direct atmospheric oxidation without the intervention of an enzyme was shown as follows. Young white rhizomes were extracted with chloroform water in an atmosphere of nitrogen as described above. The vessel was connected by a rubber tube to a distillation flask through which nitrogen also circulated. After warming for an hour at 45° the extract was transferred through the rubber tube from the extracting bottle to the distillation flask without exposure to air. The extract was then boiled vigorously over a naked flame for one minute in a current of nitrogen and, after cooling, exposed to air. The characteristic colour changes

from colourless through blue and green to yellow were obtained, showing that the phenomenon is able to take place under circumstances which preclude the possibility of enzyme action.

It has been shown that a permanent blue compound is formed by *Mercurialis* on drying [Haas and Hill, 1925]; it is now seen that the plant also yields a transient blue compound by atmospheric oxidation. The question arises whether the same chromogen is involved in each phenomenon—a precursor which by loss of water yields the permanent blue and by oxidation gives the fugitive blue. To test this the following experiment was carried out to see whether the chromogen extract, when evaporated to dryness in an atmosphere of nitrogen, could be made to yield the permanent blue.

Exp. 3. A chloroform water extract was transferred to a distillation flask as described in Exp. 2 and there evaporated to dryness under reduced pressure in a current of nitrogen. The flask contained a small quantity of a deep blue solid which, when taken up with water, and exposed to the air retained its colour unimpaired, from which it may be concluded that the conversion of the chromogen into the permanent blue had been effected by desiccation, as had been anticipated.

It will be remembered that tissues which have produced the transient blue to their fullest extent never yield the permanent blue on desiccation (p. 237). For these reasons we incline to the view that it is the same chromogen which yields either the one or the other blue compound according to the conditions, but a sufficient answer to this question cannot be given until the chemistry of the substances involved has been fully investigated.

The possible physiological significance of such an extremely labile substance as the chromogen described is of the greatest interest, but speculation at this early stage is unprofitable; much more information is requisite. Thus, before considering this substance as a respiratory chromogen in Palladin's sense, but differing from his in that enzyme action is not involved, it is necessary to ascertain whether the oxidative changes producing the blue and yellow compounds are reversible or not. In answer to this question it may be said that so far only the oxidation of the chromogen to blue has been reversed; thus, if to a solution of the fugitive blue a little sodium hydrosulphite is added the blue colour is discharged, but is restored by careful addition of hydrogen peroxide, only to be further and irreversibly oxidised to the yellow compound on the addition of excess of the reagent. Attempts to reduce the yellow compound back either to the blue or to the chromogen by means of the same or other reagents have so far not met with any success and solutions of the yellow compound treated with excess of hydrosulphite give no indication of production of the blue on carefully regulated addition of hydrogen peroxide.

These and other aspects of the subject are under investigation and our observations, together with some account of the chemical properties of the substances involved, will form the subject of a future communication.

SUMMARY.

1. In *Mercurialis* there is a chromogen which undergoes oxidation by gaseous oxygen in two stages, the first stage results in the production of fugitive blue compound which is further oxidised to a permanent yellow substance.

2. The oxidation of the chromogen is independent of enzyme action.

3. The fugitive blue is distinct from the blue produced on desiccation.

4. There are reasons for considering that there is only one chromogen concerned, which forms either the fugitive blue or the permanent blue, according to conditions.

We desire to express our indebtedness to Miss Beatrice Lee, M.Sc., for her valuable help in the preliminary phases of the investigations described in this and the previous paper, whilst working in this laboratory under a grant from the Department of Scientific and Industrial Research.

REFERENCE.

Haas and Hill (1925). *Biochem. J.* **19**, 233.

XXXV. SYNTHESIS OF VITAMIN A BY A FRESH-WATER ALGA, *CHLORELLA* (sp.?).

By KATHARINE HOPE COWARD.

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(*Received February 26th, 1925.*)

JAMESON, DRUMMOND and COWARD [1922] showed that a marine diatom, *Nitzschia closterium* W. Sm., synthesises vitamin A when grown in Miquel's solution or sterilised sea-water in diffused sunlight. This work was, however, open to the criticism that the culture, one supplied by Dr Allen, Plymouth, was not pure, having been found to contain bacteria. Hence it was decided to repeat the work on a culture of *Chlorella* (species undetermined) which was known to be free from bacteria.

Two experiments were performed. In each the culture medium consisted of:

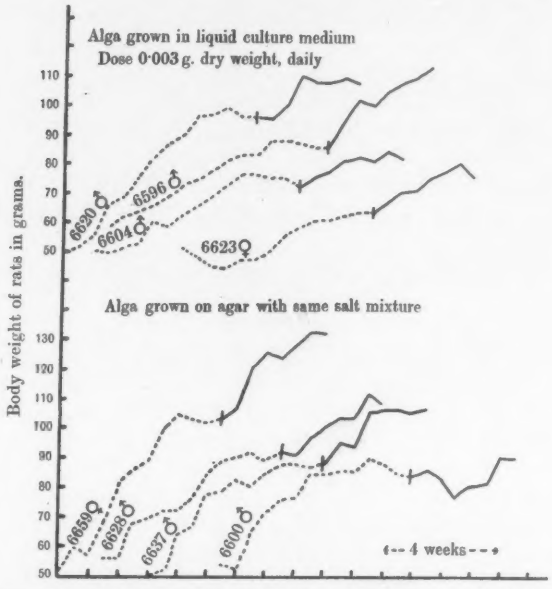
$Ca(NO_3)_2, 4H_2O$	1·7 g.	dissolved in water,
$MgSO_4, 7H_2O$	0·41 g.	
$CaCl_2$ (anhydrous)	0·123 g.	
$FeCl_3$	50 cc. 0·005 % solution	
K_2HPO_4	0·20 g. dissolved separately.	

The whole of the above was made up to 1000 cc.

For one experiment the above solution was used alone; for the other, 1·5 % agar was added to it. Four flasks of 250 cc. capacity and containing 30 cc. of the culture medium were inoculated daily in each experiment with an emulsion of the alga, for thirty days. Bacteriological precautions were used throughout. The flasks were plugged with cotton-wool, covered with caps of paper and kept near a window with a south aspect for two months in the late summer. In no case were any signs of bacterial growth observed.

For the feeding test the culture was filtered through a wad of starch in a Gooch crucible, as in the previous experiment on *Nitzschia*. In the case of the cultures on agar, the alga was scraped off, and mixed intimately with a small piece of diet. The daily dose per rat (the culture of one flask) was approximately 0·003 g. dry weight. The basal diet, free from vitamin A, was the one used in the diatom experiment and in general use in these laboratories.

Marked resumption of growth in the test animals indicated that *Chlorella* (sp.?) grown in pure culture had synthesised vitamin A.



Preparatory period on basal diet free from vitamin A shown in dotted lines

The thanks of the writer are due to Dr Roach of the Rothamsted Experimental Station for supplying the culture of *Chlorella*, and to the Medical Research Council for a grant from which the expenses of this experiment were defrayed.

REFERENCE.

Jameson, Drummond and Coward (1922). *Biochem. J.* **16**, 482.

XXXVI. THE PHOSPHATE METABOLISM IN FATIGUED MAMMALIAN MUSCLE.

By SAMUEL ANDREWS.

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(*Received February 27th, 1925.*)

The extreme importance of the changes undergone by the hexosephosphoric esters in the metabolism of muscle has only within recent years been recognised. In a long and important series of papers from Embden and his school the general nature of the changes in these esters (lactacidogen) has been gradually elucidated.

The influence exerted by the various anions on the metabolic changes, anabolic and catabolic, which lactacidogen undergoes, has been the subject of certain recent researches by Embden and Lehnartz [1924], Embden and Haymann [1924], Abraham and Kahn [1924], and Lange and Meyer [1924]. From these investigations it is evident that various anions, such as thiocyanate, chloride, etc., give rise to a disintegration of the lactacidogen molecule, whilst others, especially the fluoride and oxalate, produce a synthesis. From Embden and Haymann's work it is evident that the action of the fluoride is a true synthetic one, because accompanying the disappearance of the free phosphoric acid there is a definite formation of the diphosphoric ester. It is also evident that the process does not require the intact muscle fibre, as it is to be observed in the expressed muscle juice as well as in minced muscle.

It is extremely difficult in many cases, as Embden, Schmitz and Meincke [1921] have proved for frog's muscle, and to a lesser extent for dog's muscle, to show that the lactacidogen diminishes during activity, owing to the rapidity with which a recuperative synthesis of the carbohydrate and the phosphate takes place.

In this communication attention will be directed mainly to the different effects produced by the fluoride anion acting upon resting and stimulated muscle. It was recognised from Embden's investigations and also from many of my own, that this synthetic action might be increased by the addition of glycogen to the fluoride solution; but the effects produced by the fluoride alone are so well marked that such an addition was found to be unnecessary.

Embden, when estimating the amount of phosphoric acid already preformed in the muscle, made use of a method in which he fixed the muscle in hydrochloric acid and then precipitated the protein with mercuric chloride, removing the excess of mercury with sulphuretted hydrogen. The value of free

phosphate which he found in his filtrate he called the A value. Then he warmed the muscle with sodium bicarbonate solution in order to break down the lactacidogen. The total phosphate value thus obtained he called the B value. The difference between B and A was the amount of phosphoric acid bound as ester.

In the experiments which follow I shall make use of these letters when referring to the free and total phosphate values, and the value after the action of fluoride I shall refer to as the C value.

The resting and stimulated muscle of the rabbit, cat and dog were examined. In all the experiments on rabbit muscle the pale variety was used.

Technique.

The muscle, immediately it was taken out, was placed in a vessel cooled in ice. Small specimens (about 1 g.) were quickly minced on an ice cold porcelain slab, and placed in 5 cc. quantities of the various solutions. The protein was precipitated by the addition of trichloroacetic acid, and the muscle was finely ground up in a mortar and filtered. The extraction was repeated three times and the washings all passed through the same filter.

The inorganic phosphate was estimated in the filtrate by Embden's gravimetric method [1921], which was found in all the controls to give very accurate results. The preformed inorganic phosphate (Embden's A value) was found by taking the muscle directly into 5 % trichloroacetic acid. As will be seen, this method gives results very comparable with those obtained by the hydrochloric acid extraction method. The lactacidogen values (Embden's B – A value) were obtained by heating the muscle with 2 % sodium bicarbonate for 2 hours at 40° in each case.

Numerous controls were carried out, and duplicates were done with the majority of the specimens. It was found that the duplicates, when the conditions were similar, did not differ by more than 2–3 %.

Rabbit Muscle.

In the first three experiments resting rabbit's muscle was examined. The animals had been fed for 2 days previously with oats and carrots. Each was killed by a blow on the back of the head, and bled. Portions of pale muscle were quickly excised from the adductors of the lower limb.

	% H_3PO_4		
	Rabbit I	Rabbit II	Rabbit III
In 5 % trichloroacetic acid	0·356	0·332	0·373
In 2 % sodium bicarbonate, heated 2 hours 40° ...	0·576	0·601	0·623
In 0·9 % sodium chloride, heated 2 hours 40° ...	0·522	0·550	0·546
In *N*/10 sodium fluoride, 2 hours	0·093	—	—
3 „	—	0·057	0·100
5 „	—	0·057	—
6 „	0·044	—	—
18 „	0·118	—	—
heated 2 „	0·107	—	—
In *N*/10 sodium fluoride + 0·4 % glycogen, 3 hours 12°	—	0·012	0·057

If we take the trichloroacetic acid value as corresponding to Embden's A value and the heated bicarbonate figure as corresponding to his B value, we obtain in each case the lactacidogen value.

	Rabbit I	Rabbit II	Rabbit III
Lactacidogen	0·220 %	0·269 %	0·250 %

The preformed inorganic phosphate value does not vary much in the three animals.

On heating with bicarbonate there is a marked rise in the inorganic phosphate content, corresponding to increases of I, 61·8 %, II, 81·0 %, III, 67·0 %.

On heating with normal saline, the chloride ion is also found to exercise a disintegrative effect on the lactacidogen, although the increase is never so marked as with bicarbonate. Increases: I, 46·6 %, II, 65·6 %, III, 46·4 %.

Under the action of fluoride there is a marked decrease in the amount of inorganic phosphate; this action seems to reach its maximum in 3–5 hours.

The differences in the trichloroacetic acid value and the fluoride value are practically the same in each of the three rabbits, viz. I, 0·263, II, 0·275, III, 0·273.

When glycogen is added in addition to the fluoride, the inorganic phosphate falls to a very low level, the quantities being too small to permit of accurate determination.

Similar experiments were carried out on strychninised rabbits, and the results may be compared with those obtained in normal resting rabbits.

Rabbit IV. The strychnine was injected in increasing doses at 10-minute intervals for 45 minutes. The animal gradually became more spastic and 5 minutes after the last injection developed strong convulsions and died.

	% H_3PO_4
In *N*/10 sodium fluoride	0·263
In 5 % trichloroacetic acid	0·354
In 0·9 % sodium chloride, heated 2 hours 40°... ...	0·456
In 2 % sodium bicarbonate, heated 2 hours 40° ...	0·531

Rabbit V was injected with strychnine in small doses over a period of $3\frac{1}{2}$ hours. Ten minutes after the last injection it developed strong convulsions and died in 5 minutes.

	% H_3PO_4
In *N*/10 sodium fluoride, after 6 hours 12°	0·443
In *N*/10 sodium fluoride, after 7 hours 12°	0·440
In 5 % trichloroacetic acid	0·360
In 0·9 % sodium chloride, heated 2 hours 40°... ...	0·466
In 2 % sodium bicarbonate, heated 2 hours 40° ...	0·478

In both animals the preformed or A value is about the normal, but the B value is lower than normal. This gives a lower lactacidogen value than normal, in one case 0·177 %, in the other 0·118 %.

The fluoride values are much higher than those found in resting rabbit's muscle. In the second rabbit the fluoride value was actually higher than the A value. It cannot be the inorganic phosphate that is lacking for synthesis, since this is as high as in the normal animals.

Cohn [1921] found for the lactacidogen values in the pale muscle of the strychninised rabbit figures ranging from 0·1286 % to 0·2057 %, with an average of 0·1635 %.

The values obtained in the above experiments may be summarised as follows:

	% H_3PO_4		
	Synthesis.		Hydrolysis.
	C ←	A	→ B
Resting muscle	0·044	0·356	0·576
" "	0·057	0·332	0·601
" "	0·100	0·373	0·623
Fatigued "	0·263	0·354	0·531
" "	0·440	0·360	0·478

It is of interest to note that Embden and Adler [1921], using 10 g. of muscle, obtained for pale resting rabbit's muscle A values from 0·2008 % to 0·2755 % (average 0·2442 %) and B values from 0·5295 % to 0·6101 % (average 0·5712 %); and for lactacidogen values varying between 0·2800 % and 0·4093 % (average 0·3179 %). Using red muscle they obtained somewhat lower values.

Dog Muscle.

Effect of Stimulation.

Exp. 1. The dog was anaesthetised with ether. The sciatic nerve on one side was exposed, severed and the distal portion stimulated for 40 minutes with single induced shocks and then for 10 minutes with interrupted tetanising stimuli. The limb acted against a weight, and at the end the muscles were almost completely fatigued. The muscles in the resting and in the fatigued limbs were then exposed, sprayed with ethyl chloride and corresponding portions excised. They were placed in an ice-cold vessel and rapidly minced. Similar portions were at once placed in the three solutions and weighed.

	% H_3PO_4	
	Resting	Fatigued
In 5 % trichloroacetic acid	0·259	0·225
In 2 % sodium bicarbonate, heated 1 hour 40°	0·457	0·396
In *N*/10 sodium fluoride, 1 hour 12°	0·067	0·277

Both the A and B values of the muscle on the resting side are higher than those on the fatigued. On the resting side the B value is 76·4 % higher than the A. This corresponds to a store of lactacidogen which furnishes on complete breaking down this increment in free H_3PO_4. On the fatigued side the increment in free H_3PO_4 is practically the same although the absolute values (A and B) are down.

On the resting side the fluoride has caused a decrement in free H_3PO_4 amounting to 73·3 %. On the fatigued side the fluoride has, instead of producing a decrement in free H_3PO_4 from the A value, given rise to a 23·1 % increment. The stimulated muscle, which contains only 0·171 % of lactacidogen, is unable to synthesise in the presence of fluoride, whereas the resting muscle (0·198 % of lactacidogen) readily produces this effect.

Exp. 2. The dog was anaesthetised with ether, and one sciatic nerve was exposed and cut. A large proportion of the animal's blood was removed by perfusion with normal saline.

When the nerve was stimulated the muscles appeared to become fatigued very quickly—in about a minute. A specimen was taken into fluoride and the stimulation was continued for 5 minutes and again for 15 minutes.

	% H_3PO_4	
	Resting	Stimulated
In $N/10$ sodium fluoride	0·069	—
" " " stimulated 1 minute	—	0·121
" " " " 5 minutes	—	0·120
" " " " 15 "	—	0·126
In 2 % sodium bicarbonate, heated 2 hours 40°	0·400	0·310

The action of the fluoride was again more marked on the resting than on the fatigued side. The total phosphate (preformed and lactacidogen) showed a marked diminution (22·5 %) compared with that obtained on the unstimulated side.

In this animal some experiments were devised to find out how treatment with sodium chloride affected the fluoride synthesis in the muscles on the resting side.

Resting muscle.	% H_3PO_4
In $N/10$ sodium fluoride, 1 hour 11°	0·069
In fluoride 1 hour, then washed and placed in saline 2 hours 40°	0·124
In saline 2 hours 40°	0·603
In saline 2 hours 40°, then left overnight in fluoride	0·361
In saline 20 minutes 40°	0·238
In saline 20 minutes 40°, then in fluoride for 1 hour	0·116

The maximal disintegration of the lactacidogen occurred in the specimen kept in 0·9 % sodium chloride at 40° for 2 hours.

After a preliminary 1 hour action of fluoride followed by 2 hours' action of chloride at 40°, where the fluoride building-up action is counteracted by the chloride breaking-down action, the free H_3PO_4 is brought approximately to the same level as by the fluoride alone acting upon stimulated muscle. When, on the other hand, the breaking-down action of chloride is first allowed to take place and then the muscle is treated with fluoride, the former effect is dominant, so that the fluoride is not able to synthesise to the same extent.

Exp. 3. The dog was anaesthetised with ether. The sciatic nerve on one side was severed and stimulated for 5 minutes with interrupted tetanising stimuli. The muscles showed no evidence of fatigue in this time. Specimens were quickly taken from each side into fluoride. Transfusion was then begun and most of the animal's blood was replaced by saline. At the same time stimulation of the sciatic nerve was continued. The muscles quickly became fatigued—in about 5 minutes fatigue was complete. Specimens of muscle from both sides were then taken.

	% H_3PO_4	
	Resting	Stimulated
Before transfusion:		
In $N/10$ sodium fluoride	0·081	0·077
After transfusion:		
In $N/10$ sodium fluoride	0·071	0·148
In 2 % sodium bicarbonate, heated 2 hours 40°	0·373	0·264

The resting muscle, after heating with bicarbonate, showed a higher H_3PO_4 value (by 41·3 %) than the fatigued.

The fluoride synthetic effect was not changed by 5 minutes' stimulation prior to transfusion, but after the onset of fatigue subsequent to transfusion the synthetic effect of the fluoride was markedly lowered.

A few experiments carried out on the resting muscle alone with regard to the effect of fluoride after the action of chloride at 40° for 2 hours showed no appreciable synthetic effect at 0°.

Exp. 4. A dog was anaesthetised with ether. The sciatic nerve was exposed on one side and stimulated for 20 minutes with interrupted tetanising stimuli. At the end of this period the muscles were still contracting vigorously. Perfusion was commenced and at the same time the animal was bled from the carotid. Stimulation of the sciatic nerve was continued. Unfortunately the heart failed in about two minutes and the animal died. The muscles were quickly excised and examined in the usual way.

	% H_3PO_4	
	Resting	Stimulated
In *N*/10 sodium fluoride	0·079	0·178
In 5 % trichloroacetic acid	0·244	0·219
In 2 % sodium bicarbonate, heated 2 hours 40°	0·375	0·368
In 0·9 % sodium chloride, heated 2 hours 40° ...	0·392	0·374

Here again the H_3PO_4 value in the resting muscle treated with fluoride is much lower than that of the stimulated muscle treated in a similar way.

The A and B values are both lower in the stimulated muscle. The lactacidogen value is slightly higher in the stimulated muscle; but when acted upon by fluoride the free H_3PO_4 is diminished by 67·6 % in the resting muscle and only by 18·7 % in the stimulated.

Cat Muscle.

A cat was anaesthetised with ether. The vessels were perfused with saline and while the blood was gradually being washed out the sciatic nerve on one side was exposed and stimulated. After being tetanised for 10 minutes with short interruptions the muscles were completely fatigued.

	% H_3PO_4	
	Resting	Stimulated
In *N*/10 sodium fluoride	0·124	0·212
In 5 % trichloroacetic acid	0·315	0·278
In 0·9 % sodium chloride, heated 2 hours 40° ...	0·477	0·392
In 2 % sodium bicarbonate, heated 2 hours 40°	0·445	0·345

Both the A and B values of the fatigued muscle are lower than those of the resting, and the difference between the A and B values shows in the resting muscle an increment of 41·2 % in free H_3PO_4 and in the fatigued muscle an increment of 24·1 %. The absolute increments are 0·130 and 0·067 % respectively. The lactacidogen value of the stimulated muscle is, therefore, much below that of the resting.

The fluoride effects show a synthesis in both the resting and the fatigued, well marked in the former (0·191 %) and only slight in the latter (0·066 %).

Estimations of glycogen and in many cases of glucose and lactic acid were performed in these muscle specimens, with the object of correlating the carbohydrate changes with those of the phosphoric acid. In this preliminary communication, however, attention has been directed solely to the phosphate changes. It will be of interest to see if the addition of glycogen to the fluoride in the case of fatigued muscle can restore the synthesising power to the level normally found in unfatigued muscle.

Summary.

1. Glycogen when added to the fluoride acting on resting muscle causes practically a disappearance of free phosphate from the mixture.

2. During muscular fatigue there is a slight diminution of the total phosphate (preformed and lactacidogen) and also of free phosphate in the muscle.

3. In fatigued muscle, with practically as much preformed phosphate as in the resting, the synthetic action of fluoride is greatly diminished.

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XXXVII. THE PRODUCTION OF ACIDOSIS BY INGESTION OF MAGNESIUM CHLORIDE AND STRONTIUM CHLORIDE.

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(*Received March 2nd, 1925.*)

Haldane, Hill and Luck [1923] put down the acidosis caused by the ingestion of $CaCl_2$ to a precipitation of $CaCO_3$ in the intestine. If this is correct, similar effects are to be expected on taking $MgCl_2$ and $SrCl_2$. In the former case the reaction occurring in the gut would be:

$$MgCl_2 + 2NaHCO_3 = MgO_2H_2 + 2NaCl + 2CO_2,$$

in the latter:

$$SrCl_2 + 2NaHCO_3 = SrCO_3 + 2NaCl + CO_2 + H_2O,$$

the insoluble hydroxide or carbonate being excreted rectally, the NaCl reabsorbed. The net effect would be the substitution of $NaHCO_3$ by NaCl in the blood and tissues, just as if an equivalent of HCl had been taken. A certain amount of the bivalent metal would also be precipitated and excreted as soap, with the same effect on the reaction of the tissues.

On two occasions J. B. S. H. drank a litre of water in which 25 g. $MgCl_2$ were dissolved. The alveolar CO_2 pressure, estimated by the method of Haldane and Priestley [1905], fell from 39·3 to 32·8 mm. and from 37·2 to 31·9 mm. This fall was completed within 5 hours of taking the salt, but the pressure had not returned to normal on the subsequent mornings. The increased breathing might conceivably have been due to a stimulation of the respiratory centre by Mg; however, an analysis of the urine secreted during the 24 hours after taking the salt left no doubt of the presence of a true acidosis. While on four normal days the acid titrated to phenolphthalein varied between 57 and 61 millimols, 76 were excreted after taking $MgCl_2$. The ammonia, estimated by Malfatti's [1908] method, which had varied between 69 and 75 millimols, rose to 107. The salt caused intense diarrhoea, continuing for more than 24 hours. A sample of faeces was analysed qualitatively. The filtrate was alkaline to litmus and contained Mg and Cl. The residue, insoluble in water, was almost wholly soluble in dilute HCl. It gave no reactions for Ca or carbonate but contained much Mg. It therefore probably consisted largely of MgO_2H_2. An analysis kindly performed by Miss Watchorn showed that 0·514 g. of Mg as against about 0·14 g. on normal days had been excreted in the urine within 24 hours. The total was only 8 % of that ingested.

60 g. of $SrCl_2 . 6H_2O$ were drunk in a litre of water. The alveolar CO_2 pressure fell from 40·3 to 33·1 mm. and was still low next morning. The urine ecreted within 24 hours after taking the salt contained 68 millimols of acid and 102 of NH_3. There was therefore an acidosis. Besides diarrhoea, a slight headache, and malaise lasting for several days were experienced. The filtrate from a sample of faeces was neutral to litmus and contained much chloride, but only traces of alkaline earths. The residue insoluble in water was partly soluble in dilute HCl with effervescence. The alkaline earth metals were precipitated as oxalate and converted to nitrate. Only a trace of this was soluble in amyl alcohol. The residue gave a brilliant Sr spectrum. The insoluble part of the faeces therefore contained $SrCO_3$ and perhaps strontium soaps, but very little Ca. The alkaline earths of the urine were precipitated as oxalates, ashed, converted to nitrate, separated by extracting the $Ca(NO_3)_2$ with amyl alcohol, and estimated volumetrically as oxalate. The day's urine contained 1·46 g. of Sr, or 7·4 % of the total taken, and 288 mg. of Ca, as against a normal of 127–178. The excess is fully accounted for by the acidosis [Stewart and Haldane, 1924] and there is no evidence of a displacement of Ca by Sr.

There was a possibility that any violent diarrhoea might cause an acidosis through loss of bicarbonate. So, as a control, 40 g. $Na_2SO_4 . 10H_2O$ were drunk in 800 cc. water. This produced copious diarrhoea of a p_H about 7·5, but no change in the alveolar CO_2 pressure.

Summary.

Ingestion of $MgCl_2$ or $SrCl_2$ causes acidosis in man.

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XXXVIII. OXIDATIVE DEAMINATION BY A BASIDIOMYCETE ENZYME.

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The oxidative deamination of amino acids by tyrosinase was first observed by Chodat and Schweizer [1913], who demonstrated qualitatively that an enzyme prepared from the potato, when in the presence of oxygen and at an alkaline reaction, oxidised certain amino acids with the production of carbon dioxide, ammonia, and an aldehyde containing one carbon atom less than the original amino acid. The above reaction is stated to proceed more readily and completely in the presence of certain phenols, Schweizer [1917] considering that this acceleration was caused by the combination of the phenol with the products of the reaction. Both *p*-cresol and catechol are mentioned in this connection, but in the experiments described by these authors *p*-cresol alone was used.

The fact that this phenol is readily oxidised by tyrosinase with the production of an orange-coloured substance was first observed by Chodat [Chodat and Staub, 1907], who subsequently found that if certain amino acids were added to *p*-cresol and tyrosinase, fluorescent cresol-blue pigments were formed; he did not, however, at first ascribe this phenomenon to the oxidation of the amino acids.

Chodat and Schweizer [1915] state that it is not known whether the oxidation of tyrosine proceeds in the same manner as that detailed for amino acids such as glycine and alanine; Schweizer [1917] describes, however, the detection of small quantities of ammonia with Nessler's reagent after tyrosine had been acted upon by the enzyme in the presence of *p*-cresol.

For the work described in this paper a tyrosinase was used from the basidiomycete *Lactarius vellereus*. A crude enzyme extract was prepared according to the method described by Onslow [1920] for the phanerogams. The plant tissue was rapidly and thoroughly pounded with 97 % alcohol and was repeatedly filtered to dryness on a Buchner funnel; the residue was then extracted with phosphate buffer of p_H 7·8 for about 10 minutes and filtered, the filtrate containing a crude solution of the enzymes present, including an exceedingly active tyrosinase. The extract gave a negative ninhydrin reaction, demonstrating its freedom from amino acids and polypeptides which might have participated in the reactions, described by Chodat and Staub, between

phenols and these substances in the presence of tyrosinase. Small amounts only of the enzyme were made each time; the extracts, however, were found to retain their strength for some days if kept in a refrigerator.

The apparatus used by us to investigate the oxidation of amino acids was a modified form of the Barcroft differential manometer, four instruments being shaken mechanically side by side in a water bath at 16°, this temperature being preferred to a higher one partly because of the great rapidity of the initial oxidations, and partly to facilitate speedy equilibration rather than on account of considerations of the optimum temperature for the tyrosinase reaction. The strengths of the solutions of amino acids and phenols were those used by Chodat and Schweizer [1913], the solvent being in every case phosphate buffer of p_H 7·8.

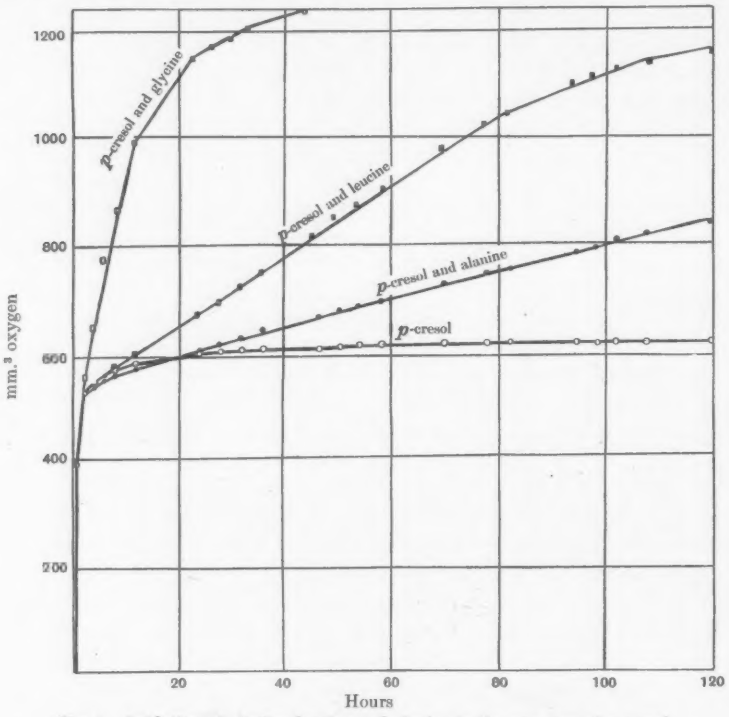

Fig. 1. Oxidation of glycine, leucine and alanine in the presence of *p*-cresol.
2 mg. *p*-cresol. 4·5 mg. glycine.
Equimolecular solutions of leucine and alanine

The enzyme extract alone was found to be entirely unable to oxidise glycine, alanine and leucine, though tyrosine showed a rapid oxygen uptake, accompanied by melanin formation. In the presence of *p*-cresol, however, the three former amino acids were readily oxidised (see Fig. 1). It will be seen that the

oxygen uptake of glycine is more rapid than that of leucine, which, in its turn is oxidised more readily than is alanine. With the enzyme preparation used, oxidation of glycine was complete in about 40 hours, the amount of oxygen used in excess of that absorbed by *p*-cresol alone being approximately that theoretically required for the equation

$$CH_2(NH_2).COOH + O \longrightarrow HCHO + NH_3 + CO_2.$$

The oxidation of leucine or alanine was incomplete after 100 hours, when the shape of the curve for leucine indicates a diminution of the velocity of the reaction, possibly due to the inactivation of the enzyme. The form of these oxidation curves seems to indicate the existence of a coupled reaction, reminiscent of that obtained in the oxidation of unsaturated fats by the sulphydryl grouping. It is noteworthy that the concentration of *p*-cresol does not appear to be a limiting factor of the velocity of the reaction; further work on the kinetics of the reaction is now in progress.

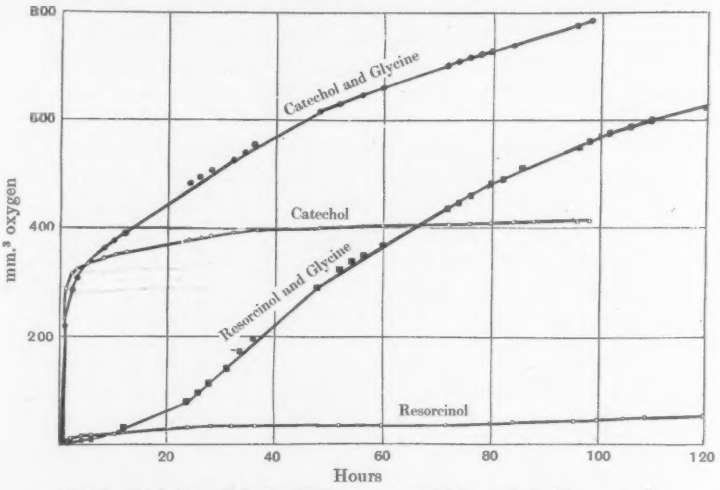

Fig. 2. Oxidation of glycine in the presence of (*a*) catechol, (*b*) resorcinol.

4·5 mg. glycine.	2 mg. catechol.
4·5 mg. glycine.	2 mg. resorcinol.

p-Cresol was then replaced by an equimolecular solution of catechol, and again oxidation of glycine was obtained. The initial oxygen uptake of catechol when catalysed by the *Lactarius* enzyme was extremely rapid, therefore a special form of bottle was used on the Barcroft manometer, a measured volume of the enzyme extract being introduced, from a side burette fitted into the bottle, after equilibrium had been attained; after addition of the enzyme the taps were immediately turned so as to connect the bottles and the manometer. The result of this experiment is shown in Fig. 2. It will be seen that

the oxidation of glycine in the presence of catechol is less rapid than in that of *p*-cresol, when an enzyme extract of approximately similar strength is used, oxidation being incomplete in 100 hours. For these experiments Merck's resublimed catechol was used; it may be noted that one molecule of catechol upon oxidation takes up one molecule of oxygen.

Using the same technique, one of us (M. E. R.) has shown that glycine and leucine are oxidised in the presence of *p*-cresol and a potato oxidase, the method of preparation of the enzyme being the same as that described for *Lactarius*. Glycine is also oxidised in the presence of catechol. The curves obtained are similar to those given for the oxidations in the case of *Lactarius*.

p-Cresol and catechol were then replaced by resorcinol. The reaction in the presence of this phenol is somewhat different, oxidation taking place only after a latent period which sometimes extended to 15 hours, the phenol itself without the amino acid in the presence of the enzyme taking up practically no oxygen during this period. Resorcinol, unlike catechol and *p*-cresol, is not autoxidised if allowed to stand exposed to the air for a considerable period, manifesting at the end of this time no peroxide formation on testing with guaiacum and horse-radish peroxidase.

The enzyme extract was found to be unable to oxidise glycine in the presence of carefully buffered solutions of *p*-hydroxybenzoic acid or aldehyde, nor were these substances themselves oxidised. Similarly the oxidation of protocatechuic acid and aldehyde was extremely slow in comparison with that of catechol, though there was evidence of oxidative deamination of glycine in the presence of the former.

Tyrosine itself is unable to replace *p*-cresol in the oxidation of amino acids. When glycine was added to a saturated solution of tyrosine in phosphate buffer of p_H 7·8, the oxygen uptake was no greater than that of a similar solution of tyrosine in the absence of glycine; on the other hand, no inhibition of the speed of oxygen uptake of tyrosine when in the presence of other amino acids was observed.

The decrease of amino nitrogen at the close of the reaction was investigated by means of the micro form of Van Slyke's apparatus. The solutions were removed from the bottles of the Barcroft manometer by means of a pipette, and the amino nitrogen content was compared with that of a control solution which had been prepared with boiled enzyme and which had been shaken concurrently in another Barcroft apparatus.

Results obtained were as follows:

Substance	O_2 uptake of amino acid at 16° mm.³	Amino N of control mg.	Amino N after deamination mg.	Loss of amino N mg.	Theoretical loss calc. from O_2 uptake mg.
Glycine + *p*-cresol	640	0·844	0·054	0·790	0·800
Leucine + *p*-cresol	540	0·875	0·265	0·610	0·628
Glycine + catechol	380	0·842	0·346	0·496	0·509

A decrease of amino nitrogen, and the production of ammonia, was observed also in the case of glycine and resorcinol in the presence of the enzyme.

The results of estimations of the ammonia produced during the course of the reaction did not correspond exactly with the observed loss of amino nitrogen, giving slightly lower values. Estimations of the total nitrogen content, by Kjeldahl's method, before and after oxidation had taken place, showed however that no loss of nitrogen as ammonia from the alkaline fluid had occurred. It is possible that the aberrant nitrogen may have taken part in the production of the coloured complex.

That Chodat and Schweizer observed oxidation of amino acids by tyrosinase alone, though acceleration of the reaction was obtained in the presence of certain phenols, may possibly be explained by the supposition that the enzyme extract used by them, prepared from the potato, may have contained traces of an autoxidised *ortho*-dihydroxy compound [Onslow, 1920]. Schweizer states that he prepared tyrosinase according to the instructions given by Chodat in Abderhalden's *Handb. d. biochem. Arbeitsmethoden*. The preliminary stages of this method include mincing of the potatoes and maceration with water of the crude alcoholic precipitate containing the enzyme, during which processes a certain amount of oxidation is difficult to avoid. Autoxidised substances might possibly be adsorbed on the proteins present together with the enzyme, and be unremoved by subsequent reprecipitations.

The basidiomycete *Lactarius vellereus* does not appear to contain *o*-dihydroxy substances [Robinson, 1924]; the enzyme extracts used in our experiments, moreover, did not undergo spontaneous darkening or oxidation on being shaken in air for a considerable period.

Summary.

A crude enzyme extract, containing tyrosinase, from the basidiomycete *Lactarius vellereus* is unable to oxidise the amino acids, glycine, leucine and alanine unless certain phenols be present. Oxidative deamination of glycine has been brought about in the presence of *p*-cresol, catechol and resorcinol. *p*-Cresol cannot be replaced in this reaction by *p*-hydroxybenzoic acid or aldehyde.

A possible explanation for the divergence of our results from those of Chodat and his co-workers is given in the text.

In conclusion we wish to express our thanks to Sir F. G. Hopkins, F.R.S., and to the Hon. Mrs Onslow for their continual interest during this work and for much valuable advice.

Note [by R. A. McCance]. Although neither *p*-cresol nor resorcinol decolorises methylene blue either alone or in the presence of tyrosinase, the system, glycine (or any amino acid) plus *p*-cresol, resorcinol, etc., plus tyro-

sinase has been found to decolorise methylene blue freely. Of the amino acids tried, glycine is the most active in this connection. Tyrosine can act in this connection in its capacity as an amino acid.

A study of this reaction has been made with a number of substances and the results and conclusions as to the nature of the chemical changes involved will be published shortly.

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XXXIX. THE RELATION OF PECTOSE AND PECTIN IN APPLE TISSUE.

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It has long been maintained that pectose, or proto-pectin as it is called by some authors, is an insoluble constituent of plant tissues, associated with cellulose in the cell-walls but exhibiting none of the properties of that substance. It has also been claimed by the majority of investigators that during the ripening of fruit, pectose decomposes into a soluble form, generally known as pectin, which consequently appears in increasing amounts in the expressed cell-sap as the fruit matures. In the unripe fruit, however, pectin occurs in negligible quantities only.

Concurrently with this disturbance in the pectic constituents, the fruit exhibits signs of softening as ripening proceeds. Microscopic examination of the tissues shows that this softening is associated with structural relations and staining and chemical reactions of the cell-wall which can only be interpreted as due to the conversion of an insoluble substance like pectose into soluble pectin.

In advanced stages of over-ripeness and during senescence the pectic changes become very much pronounced. In this condition the tissues are readily disintegrated, the cell-walls become markedly thinner, and the cells ultimately separate from one another owing to solution of their middle lamella substance.

It has been found possible to locate the pectic substances in the cell-walls by staining with ruthenium red, and to follow their distribution throughout the development of the fruit[1]. Pectic substances have been located in the cell-walls and middle lamella, and periodic examinations of the apple tissue as the season advances confirm the view that the physical modifications in mature fruit are due to pectic changes, and that the processes of ripening and physiological breakdown are intimately associated with the progressive change of an insoluble pectic substance into a soluble modification. As a result of chemical work already published in part [Carré, 1922] and the unpublished

[1] This work has been carried out in conjunction with Dr A. S. Horne and will shortly be ready for publication.

microscopical studies referred to above, it would seem difficult to hold any other view than that pectose (proto-pectin[1]) is chemically different from the pectin to which it gives rise, both these substances occurring in the tissues of ripe fruit.

An exactly opposite view, however, has been put forward by F. Tutin [1923], who vigorously disputes the conception of pectose and pectin as chemically different substances in the tissues, and upholds an alternative explanation of the phenomena to be observed during the ripening processes of fruits.

Tutin regards the existence of pectose as "purely hypothetical," and considers that the pectic material of the tissues consists of pectin only, which is "held in its typical soluble form throughout the development of the fruit." He maintains that ripening is in no way associated with chemical changes in the pectic components of the tissues, but that the apparently insoluble condition of the pectin (especially in hard unripe fruit), which is usually explained by the existence of pectose, is really due "to the great difficulty in attaining by mechanical means, really efficient disintegration of the hard unripe tissue."

Tutin therefore suggests that extraction with water alone should be sufficient to dissolve out the whole of the pectin thus held in the tissue, provided that complete disintegration of the material can be obtained. He consequently regards the ease with which pectin is extracted from ripe fruit as compared with unripe to be solely due to alterations in the physical condition of the fruit, and in no way connected with any chemical change in the pectic constituents themselves. Tutin also suggests that apart from the difficulties of mechanical disintegration, another factor is at work, namely, the action of certain constituents of the tissues, which tends to retain the pectin and prevent its ready removal with water. In support of this, he states that solutions of pectin approximating to a concentration of 0·5 % cannot be filtered without undergoing change in concentration, owing to retention of some of the dissolved pectin by the filter paper. He suggests that when attempts are made to extract the pectin from the tissues by treatment with water, the cell walls retain pectin in a similar manner.

It would seem impossible, however, to accept such a view. The question of the retention of pectin during filtration through various media has been investigated by the author, and it has been found that the amount absorbed varies directly with the concentration of the solution employed, but that solutions containing less than 0·9 to 1·0 % of pure pectin exhibit no alterations when filtered through ordinary filter paper. If on the other hand the solution to be filtered has a concentration greater than 1·0 % of pectin, an appreciable amount may be taken up. Such concentrations of pectin are however greater than that obtained in normal juice or under the experimental conditions of the usual extraction methods.

[1] As the word "pectose" receives the support of the majority of workers in this field, it is here adopted in preference to "proto-pectin."

These results are illustrated by the following examples:

Effect of Filtration on Pectin Solutions. Concentration of Pectin Solution (100 *cc.*).

	Before filtration		After filtration	
		%		%
1	...	0·025	...	0·025
2	...	0·115	...	0·116
3	...	0·227	...	0·226
4	...	0·760	...	0·759
5	...	0·870	...	0·873
6	...	0·170	...	0·148

Experimental evidence has also been obtained showing that pectin solutions of concentrations similar to the above are unchanged by passage through a dense mass of tissue from which pectin had been previously removed by water. Since the methods of extraction of pectic substances involve the use of a large bulk of water, the concentrations of pectin rarely exceeding 0·05 %, such solutions will be unchanged by filtration through paper or in their passage through the tissues themselves. It is therefore evident that the holding back of pectin cannot be regarded as responsible for the supposed retention of the pectic constituents of the unripe tissues.

Tutin describes two further experiments which he considers afford conclusive evidence for his views.

In an earlier communication, Carré [1922] showed that pectose and pectin could be removed separately from apple tissues by first extracting the soluble pectin with *cold* water, and subsequently treating the residue with dilute hydrochloric acid, thus converting pectose into pectin which can then be removed with cold water as before. Tutin has carried out a similar experiment but interprets it very differently. He treated three equal quantities of apple residue obtained during the production of cider. One sample was exhaustively extracted with boiling water in a Soxhlet apparatus, and 1·4 g. pectin was thus obtained. A second sample was autoclaved at 110° with dilute acid for 1 hour and subsequently extracted with boiling water as before. The extract yielded 1·6 g. pectin.

Tutin assumes that if both pectin and pectose had originally been present in the tissues, the water extraction would have yielded soluble pectin only, whereas the subsequent acid treatment should have produced a much larger amount, due to the decomposition of pectose. Since the weight of pectin in both cases was about the same, Tutin concludes that no pectose can have been present in the samples.

In criticism of these deductions the following observations may be made: (1) The material used for the experiment was the "pomace" or dry press-cake from the cider press. It is therefore obvious that the juice expressed from the apple during the production of the cider would have carried away with it the soluble pectin originally present in the fruit. Hence Tutin's experiment represents nothing more than two different methods of extraction of the

insoluble pectose remaining in the residue after the removal of the soluble pectin. (2) It has been proved by the present writer that *cold water only* can be used to extract the soluble pectin from tissues, since extraction with hot water induces slow conversion of the pectose into pectin, presumably by a process of hydrolysis analogous to that effected by dilute acid. It follows therefore that the 1·4 g. of pectin obtained by Tutin with boiling water actually represents a portion at least of the pectose present in the original sample. Traces of acid remaining in the "pomace" would also tend to accelerate the decomposition of the pectose. (3) The method of extracting pectin by autoclaving with hydrochloric acid at 110° has been found invariably to give unsatisfactory results, probably owing to the fact that such treatment tends to bring about the decomposition of the pectin thus produced. The method finally adopted for the quantitative extraction of pectose depends upon boiling the material with $M/100$ hydrochloric acid and this process is found to give reliable results.

The following experiment demonstrates the accuracy of this method as compared with the autoclave method employed by Tutin. Ten uniform samples of apple pulp treated with $M/100$ HCl at 100° gave a mean pectose yield of 0·76 %, while ten similar samples of material extracted by the autoclave method gave a mean of 0·67 % pectose, showing that the results obtained by the latter method tend to be too low.

This indicates that the 1·6 g. of pectin obtained by Tutin from his second sample using the autoclave method is also too low a result and does not represent the actual pectose content of the sample. It would also seem that the evidence derived from this experiment is insufficient to justify his assertion of the non-existence of pectose.

In his second experiment Tutin describes successive extractions carried out in four stages of pectin from unripe apple tissue. (I) A weighed quantity of dried material previously soaked in alcohol was pounded in a mortar and soaked for twenty-five successive periods of 24 hours each, the volume of water used for each extraction being 1300 cc. The resulting solutions of pectin were evaporated under diminished pressure and estimated. The pectin content of each of the last ten extracts was small and apparently constant. Tutin considers this slowing down in the removal of the pectin to be due to the resistance offered by the undisintegrated portions of the material. (II) The material was next dried and thoroughly ground with sand in order to increase disintegration and the residue was extracted as before till only negligible quantities were obtained. (III) The process of grinding with sand and extracting with water was repeated. The residue examined microscopically showed that disintegration had been very thorough, although groups of cells were observed to have escaped the grinding process. Tutin therefore concludes that a small amount of pectin necessarily remains in the residue. He suggests that complete disintegration "would be most readily accomplished by resorting to the treatment with dilute hydrochloric acid employed by Carré

for the hydrolysis of the supposed protopectin[1]" (pectose). (IV) Tutin therefore treated the residue with *M*/20 HCl in an autoclave at 110° and the resulting solution contained very little pectin. It is probable that the yield is too low in consequence of the method of procedure. Subsequent treatment with hydrochloric acid by this method produced no further yield of pectin.

The whole pectic constituents of the tissues having been removed with water alone, Tutin therefore concludes that water-soluble pectin is the sole substance of pectic nature in the tissues, and that the problem of estimating the pectin content of fruits depends mainly on complete disintegration of the tissues in order to facilitate its extraction by water.

The explanation put forward by Tutin is the one which suggested itself to the writer in the earlier stages of pectin work, but it has been found totally inadequate to interpret the various facts observed during the course of a more exhaustive research on the pectic constituents of apples. Experiments similar in procedure to those described by Tutin but of a more searching nature had already been devised before the publication of his results. These experiments afford conclusive evidence for the existence of pectose and pectin as definite chemical entities, and suggest that although mechanical disintegration is an important factor in the extraction of pectin, the whole problem cannot be reduced to such simple terms.

In criticism of Tutin's deductions it may be suggested that pectin is not originally present as such in the tissues as he supposes, but arises from pectose by a process of hydrolysis, the amount of pectin produced by any method of extraction depending on the efficacy of the hydrolytic agent employed. Hence treatment of the tissues with cold water, as Tutin describes in the foregoing experiment, will convert pectose into pectin by an extremely slow process of hydrolysis. This would account for the long period of time, approximately 40 days, which he found necessary to extract by means of cold water the whole of the pectin from his sample of apple.

Tutin accounts for the increased production of pectin as a result of grinding the material by assuming that increased disintegration favours the dissolving out of the soluble pectin, but it must be pointed out that grinding will also promote increase of hydrolysis and therefore increased yields of pectin by bringing fresh supplies of pectose in closer contact with the water. When the tissues are subjected to a more vigorous process of hydrolysis—for instance by treatment with water at 45°, or by the use of dilute acids or alkalis—the production of pectin is found to increase according to the efficacy of the agent employed. The accompanying table illustrates this point. Approximately similar yields of pectin were obtained from each, but the time required for the operation varied markedly with the hydrolytic power of the reagent.

[1] It is difficult to understand why weak hydrochloric acid should be assumed to possess a disintegrating action on the tissues apart from chemical action. As a matter of fact such acid does not produce complete disintegration, but mere separation of the cells (*vide*, p. 264).

Method	Yield of pectin g.	Time required for operation hours
Cold water	0·80	600
Water at 100°	0·795	96
M/100 HCl at 45°	0·81	24
M/100 HCl at 100°	0·80	15

The following experiments (A and B) may also be quoted in support of the hydrolysis theory outlined above.

Details of Experiment A (Fig. 1).

100 g. of apple tissue was pressed in a hand press and washed with cold water to remove any soluble pectin initially present in the tissues. The total weight of pectin thus obtained in the three fractions amounted to 0·0400 g., the final fraction containing only a minute trace of pectin (0·0005). The volume of water used for the extractions on this and subsequent days was maintained at 500 cc. in order that the pectic content of successive fractions might be properly compared. The residue was then soaked in water for 24 hours and the pressing and washing repeated. It will be seen that this treatment produced an amount of pectin (0·0415 g.) as large as the yield on the previous day, although no mechanical disintegration had been resorted to. This marked development of pectin may be attributed to hydrolysis of pectose in consequence of: (*a*) the prolonged contact of the tissues with water, (*b*) the action of enzymes liberated from the cells crushed during the pressing operations, (*c*) the accelerating effect of any acid left in the tissues, and probably to a combination of all these factors.

Further extractions of the material (3rd–6th day) produced decreasing yields of pectin, the amount obtained on the 6th day being negligible. Grinding the residue with sand (7th and 8th day) resulted in a further small amount, probably in consequence of the exposure of fresh quantities of pectose to the hydrolytic action of the water.

If the extraction of the pectin was simply a matter of solution of the occluded material by the water, the grinding operations should have produced a far greater effect than was actually observed to be the case, since subsequent operations showed that approximately two-thirds of the total pectic material still remained in the tissue. This method of procedure was discontinued on the 8th day. A point was not reached at which no further amounts of pectin were produced, since hydrolysis with cold water, although extremely slow, is a continuous process. Microscopic examination of the residue at this stage showed that the tissues were almost entirely disintegrated, but staining with ruthenium red revealed substances of pectic nature abundantly present in the fragments of cell-walls and in the middle lamella regions. In order to hasten hydrolysis the residue was re-ground with sand (8th day) and boiled with water for 5 minutes. The amount of pectin then produced (0·0900 g.) was twice as great as that obtained on the first or second day of the experiment,

and nine times as much as the amount produced by cold water alone in the preceding fraction.

The boiling was repeated daily till only small traces of pectin were extracted, the final fractions being approximately constant in amount. It will be seen from the table that the total weight of pectin obtained by boiling the residue with water is 0·4515 g., nearly three times the amount produced by cold water treatment.

It might be suggested on Tutin's theory that the large production of pectin with hot water is due to its greater solubility at the higher temperature, but experimental evidence indicates that the solubility of pectin is not appreciably greater in hot than in cold water. Neither can the production of

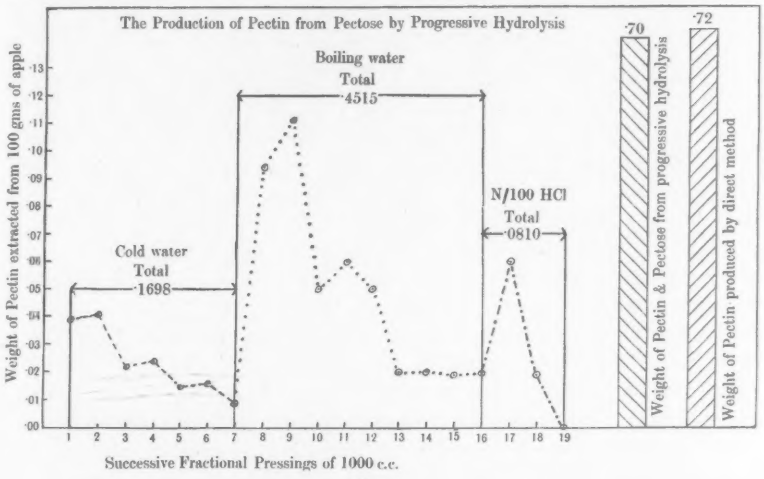

Fig. 1.

pectin be due to enzyme action in this case since the temperature of extraction is destructive to enzymes. It seems probable therefore that the enormous and rapid development of pectin can only be attributed to the increased hydrolytic action of the water on the pectose at the higher temperature.

This conclusion is borne out by the further marked increase which was produced when the residue was treated (18th–20th day) with a more powerful hydrolytic agent, *i.e.* boiling dilute hydrochloric acid (*M*/100) for 15 minutes. After two such treatments no further trace of pectin could be obtained, and microscopic examination of the residue treated with ruthenium red showed that all the pectin constituents of the tissues had been removed. This effect of the acid in removing the pectin cannot be attributed to solvent action since pectin is only very slightly soluble in dilute acids. The above experiment suggests that if dilute hydrochloric acid be used for the hydrolysis of the pectose from the outset, the extraction should be effected more rapidly than with water alone.

Experiment B.

A second experiment was therefore carried out on a 100 g. sample of apple, similar to that used in Exp. A. The pulp was extracted with $M/100$ HCl at 100° for successive 3-hour periods. After each treatment the residue was pressed and washed to remove the bulk of the pectin produced. It was found that after three such extractions no further pectin was obtained. The total extracted was 0·72 g., which agrees very closely with the 0·70 g. obtained from similar material in Exp. A, allowing for the experimental error necessarily involved in the latter (see Fig. 1).

Experiment B.

100 g. apple tissue. Material extracted with $M/100$ HCl at 100°.

Extraction	g. of pectin obtained
1	0·4810
2	0·1943
3	0·0448
4	0·0000
Total pectin ...	0·7201

Microscopic examination of the residue showed that all the pectic material had disappeared, although the tissues were not so markedly disintegrated by the acid as by the grinding with sand in the former experiment. It was found that the cells were separate from one another, in consequence of the solution of their middle lamellae, but the cell-walls themselves were almost invariably unbroken, and preserved their original outline.

Microscopic investigations of the pectic substances have been carried out by the author in conjunction with Dr A. S. Horne, and the results, which will be briefly alluded to, confirm the opinions arrived at from the chemical side. The decomposition of the pectic substances has been followed under the microscope by treating in various ways sections stained with ruthenium red, which colours the pectic constituents. Irrigation of these sections with water produced considerable swelling of the stained areas followed by their very slow disappearance. This process was observed to be much more rapid with hot water, and very much accelerated when either dilute acid or alkali was employed. Solution of the middle lamella and ultimate separation of the cells were attained, but complete removal of the pectic components of the cell-walls involved no fundamental alteration in the appearance of these walls.

It was also found that if sections were irrigated with dilute ammonium oxalate solution, which has long been recognised as a solvent for pectic substances, the solution of the stained areas was very rapidly effected. The cells were again observed to separate from one another, but the cell-walls themselves remained entirely unbroken.

It is therefore apparent from the foregoing discussion of results obtained by the author, that Tutin's theory of the non-existence of protopectin, or pectose, cannot be supported by experimental evidence. It may be concluded,

on the other hand, that pectose does actually exist as a practically insoluble constituent of the cell-walls of apple tissue, and that it can be converted into the soluble modification, known as pectin, by the action of water and by dilute acids or alkalis, the nature of the change being probably hydrolytic.

It is also evident that although mechanical disintegration is important in facilitating the process of hydrolysis, it is not, as Tutin claims, the essential factor in the removal of the pectic constituents of apple tissues, the necessity for disintegration being obviated if a sufficiently powerful reagent is used for extraction purposes.

SUMMARY.

It is shown by the author that Tutin's views on the non-existence of protopectin (pectose) cannot be maintained by the evidence afforded by his experiments. On the contrary, chemical investigations, borne out by microscopical work, indicate the presence of an insoluble pectic constituent in apple tissue, which is referred to as pectose, and which is converted by a process of hydrolysis into the soluble modification known as pectin.

This investigation has been carried out for the Food Investigation Board of the Department of Scientific and Industrial Research. The author wishes to express her thanks to Prof. V. H. Blackman and Dr D. Haynes of this department for their advice and criticism.

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XL. STUDIES ON THE METABOLISM OF ARGININE AND HISTIDINE.

PART I. ARGININE AND HISTIDINE AS PRECURSORS OF PURINES.

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The experiments described in this paper were undertaken in the hope of confirming the suggestion, advanced by Ackroyd and Hopkins [1916], that arginine and histidine are utilised in the animal body as precursors of purines. Not only did Abderhalden and his collaborators [1909, 1910], in testing this very idea, fail to obtain evidence in support of it, but since 1916 the validity of Ackroyd and Hopkins' conclusions has been called in question by several workers. Thus Lewis and Doisy [1918], maintaining normal men on purine-free high-protein diets of high and low content of arginine and histidine, found that no differences in the excretion of purines followed the ingestion of the two types of diet. More recently, Christman and Lewis [1923] have observed a marked diminution in the daily elimination of allantoin following the feeding of amino acids (glycine, alanine and glutaminic acid) to rabbits—results in agreement with those of Abderhalden and Einbeck [1909], who found the excretion of allantoin decreased when histidine was fed to dogs. On the other hand, Harding and Young [1919], feeding placenta (which is rich in arginine and histidine) to young dogs, with a control diet containing an equivalent amount of muscle protein (which is poor in these amino acids) found that the uric acid and allantoin excretions were parallel, and were markedly increased during the placenta feeding.

Not only does there remain some doubt as to whether arginine and histidine actually do function as purine precursors, but the whole of the evidence, both for and against, seems to have been derived from feeding experiments. In these circumstances, the author considered that some useful results might be obtained by employing the method of liver perfusion, in which the use of the single organ eliminates certain of the complications inherent in the use of the intact animal.

It is not necessary, of course, that a substance should be metabolised along one line only, and indeed, we have abundant examples to the contrary. Hence other suggestions as to the fate of arginine and histidine need not be considered contradictory to the hypothesis that they are or may be utilised in the syn-

thesis of purines. In the case of arginine, Thompson [1917] has shown that hydrolysis to urea and ornithine takes place, and Felix [1923] claims that when arginine is perfused through the surviving liver, this hydrolysis is nearly quantitative. Dakin and Wakeman [1911] have demonstrated the production of acetoacetic acid; and Inouya [1912], Thompson [1917], and Gross and Steenbock [1921] have adduced evidence in support of the view that arginine is a precursor of creatinine. As to the changes undergone by histidine, there seems to be no suggestion other than that of Ackroyd and Hopkins [1916]. Its capability of conversion to acetoacetic acid [Dakin and Wakeman, 1911] and to glucose in the diabetic organism [Dakin, 1913] have been examined, but in both cases with negative results.

Experimental.

The perfusions were carried out with excised cat livers in the apparatus designed by Dixon [1922], Ringer's solution being used as the perfusion fluid.

The Control. One great objection to the perfusion method is the difficulty of obtaining an adequate control. If, as is usual, separate livers are used for the positive and control experiments, the comparison can only be valid when the number of perfusions is so large that the statistical method may fairly be applied to the results. The labour involved in so large a number of perfusions is necessarily very great, and an attempt has been made in the experiments here described to secure an efficient control and yet obtain a definite result with a relatively small number of perfusions.

Each liver was perfused for a definite time with a known volume of Ringer's solution only. This fluid was then removed and replaced by an equal volume of Ringer's solution to which had been added the substance under examination, the p_H and osmotic pressure being adjusted to the normal value. This was perfused for the same length of time with, of course, the same rate of flow and the same means of aeration—a slow stream of oxygen. Estimations were carried out on both perfusates. It remained to be shown that this procedure provided an *efficient* control—*i.e.* that any increase of the substance estimated was really due to the substance perfused. A series of experiments was therefore carried out in which the liver was perfused as before with two successive equal volumes of Ringer's solution to neither of which, however, was added any other substance. Estimations were carried out as before, and it was found that there was no increase in the second perfusate—in fact the second usually gave a slightly lower result than the first. This was true not only of allantoin, but also of urea, acetone bodies and sugar. It may fairly be considered, therefore, that this method of double perfusion affords an adequate control, each positive perfusion being compared with the corresponding blank. In this way it is possible to obtain a definite and reliable result with only some half-dozen perfusions.

The Criterion. The allantoin production was taken as the criterion of purine formation since allantoin has been amply shown to be the normal

end-product of purine metabolism in the lower animals [*e.g.* Ackroyd, 1910, 1911, 1914]. It would seem that any increase in the end-product would be accompanied by an increase in the amount of each intermediate, so that in the case of purines, the more easily estimated uric acid might be used as the test-substance. Unfortunately, however, the colorimetric methods of estimating uric acid, which are best adapted for dealing with the small amounts here present, are not applicable in the presence of certain iminazole compounds which themselves give a blue colour with the various uric acid reagents.

Method of Estimation. The method of estimation was that of Wiechowski [1913] modified at the end to remove small quantities of iminazole compounds which escape precipitation in the earlier stages and are then thrown down by the allantoin reagent. Allantoin condenses with xanthydrol [Fosse and Heuille, 1923], while histidine does not, at any rate under the same conditions. The amount of condensation product isolated never corresponded to the whole of the allantoin, but, under strictly-defined conditions, it was always possible to isolate a fixed proportion.

A weighed amount of allantoin, mixed with histidine, was dissolved in the minimum amount of water and an equal volume of glacial acetic acid was added to the cold solution. Xanthydrol, in amount equal to twice the weight of the mixture (sufficient if the "mixture" was really pure allantoin), was dissolved in a volume of glacial acetic acid equal to that added to the allantoin solution; the two cold solutions were mixed, and allowed to stand for half an hour. Less time gave a smaller yield, more gave an impure product. The condensation product was then filtered off, washed four times with 5 cc. of cold water, dried at 100°, and weighed. As shown in the table below, this product was pure and corresponded to 90 % of the allantoin.

Weight of allantoin	·100 g.	·100 g.	·107 g.	·361 g.
Weight of histidine	—	·220 „	·250 „	·342 „
Weight of condensation product	·214 „	·214 „	·231 „	·777 „
Melting point of product (pure product melts at 214°) ...	214°	215°	213°	214°
% of allantoin recovered ...	89·9	89·9	90·0	89·8

In applying this method, the liquid, after perfusion, was made faintly acid with acetic acid, boiled and filtered from the precipitated protein. A measured volume of the filtrate was treated exactly as described by Wiechowski [1913]. After the addition of the allantoin reagent the liquid was allowed to stand overnight. The precipitate was then filtered off, suspended in hot water, and decomposed with hydrogen sulphide. The liquid was evaporated to dryness on the water-bath and the residue was thoroughly extracted with hot water. The combined extracts were evaporated to dryness and the residue was weighed. It was then dissolved in water and the solution evaporated to about 0·5 cc. An equal volume of glacial acetic acid was added and, after cooling, an equal volume of glacial acetic acid containing xanthydrol in amount equal to twice the weight of the residue. The condensation product of allantoin and xanthydrol was prepared as already described, its purity being

tested by determination of the melting point. Throughout the estimation equal volumes of control and positive fluids were used and the liquids received exactly similar treatment. Thus the final weighings, without any calculation, gave a strict comparison of the amounts of allantoin in the two fluids, though, of course, not the absolute amounts.

Results and Discussion.

The results of one typical perfusion are given.

	Control	Positive
Time of perfusion	2 hours	2 hours
Volume of liquid	500 cc.	500 cc.
Volume taken for estimation ...	450 „	450 „
Weight of condensation product	37·0 mg.	37·6 mg.
Melting point	214°	214°

The perfusion of arginine or histidine through the excised liver caused no real increase in the production of allantoin. The slight increase shown in the experiment quoted is within the limits of experimental error. The results are therefore disappointing in that they fail to confirm the hypothesis that these amino acids are precursors of purines. They are, however, not sufficient of themselves to invalidate the hypothesis.

Summary.

The method of liver perfusion has failed to afford evidence in support of the hypothesis that arginine and histidine function in the animal body as purine precursors.

A method of controlling liver perfusion experiments is described.

A method is given for the estimation of allantoin in presence of iminazole compounds.

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XLI. A METHOD OF MICROTITRATION.

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During a research to be published later it became necessary to obtain a continuous series of blood ammonia determinations on myself or other human subjects, but this object presented the serious difficulty that, according to the methods at present in use, about 10 cc. of blood would be required for each determination. I therefore attempted to devise a method in which only 1 cc. of blood should be required. One of the factors which make the determination of blood ammonia so difficult is that the quantity is extremely small.

The amount of ammonia in 100 cc. of blood is supposed to lie between 0·1 and 0·2 mg., which means that 1 cc. of blood can yield only 0·001 or 0·002 mg. NH_3 or about 0·1 mm.^{3} of a normal solution.

It would be quite impossible to titrate this amount by any ordinary method, but, on consideration, I found that it would not only be possible to titrate the ammonia from 1 cc. of blood, but that by a suitably arranged microtitration it could be done with far greater accuracy than the amount from 10 cc. is titrated by a macrotitration.

As I think that the method of microtitration arrived at will make all titrations of small quantities much more accurate than is possible with ordinary macrotitrations, the method and the theory upon which it is based are published separately here.

The object of a titration is to measure how much of a solution of known concentration one must add to a certain volume of another solution of unknown concentration in order to bring a certain reaction between the two fluids to completion.

This involves two subjective estimations by the person who titrates: first to judge that the reaction is completed, secondly to measure the fluid added. That the reaction is completed is judged by means of an indicator[1]; the measuring is done by means of a burette.

The errors involved in a titration are, as in other cases, partly accidental, partly systematic. The accidental errors are mainly two: first, a misjudging of the colour of the indicator, so that the p_H wanted as end point is not reached or is passed—usually one may expect the actual end point to lie within 0·3 p_H-unit on each side of the p_H wanted. The second accidental error is due

[1] In the following I shall speak only of acidimetric and alkalimetric titrations, though the reasoning will hold also for the other forms. For a complete discussion of the theory of these titrations see Bjerrum [1914].

to the reading of the burette and depends mainly on the construction of this piece of apparatus.

The systematic errors depend on the fact that free acid and alkali are still present in the solution when the end point is reached. The acid and alkali are present partly as ions and partly in undissociated form. The magnitude of the errors depends on the p_H of the end point, that is on the indicator used, on the volume of fluid at the end of the titration, and on the dissociation constants of the acid and alkali involved.

If, for instance, we titrate 10 cc. of $N/10$ acid, diluted to 90 cc., with $N/10$ alkali, and as end point use the point where methyl orange just begins to change colour (p_H 3)—we shall have at the end point 99 cc. of a p_H of 3 or 99 cc. $N/1000$ acid, corresponding to approximately 0·1 cc. N acid. If we titrate to p_H 4 the error will be only 0·01 cc. of N acid.

If we titrate the 10 cc. of $N/10$ acid directly with $N/10$ alkali to p_H 3, the error will be 0·02 cc. N acid.

For this error due to the H ions present in the solution at the end point of the titration we may give the following formula:

$$H^{\cdot}\text{-error} = C_{H^+} \times v \text{ cc. } N \text{ solution}$$

where C_{H^+} is the $H^{\cdot}$-concentration and v the volume at the end of the titration.

For the OH-ion-error we have the analogous formula:

$$OH'\text{-error} = C_{OH^-} \times v \text{ cc. } N \text{ solution}$$

where C_{OH^-} is the OH′-concentration at the end of the titration.

These two errors therefore depend on the p_H of the end point and on the volume. To keep both of them as small as possible one would have to titrate to p_H 7.

If we titrate to a p_H on the acid side of p_H 7, then the OH-ion-error is small and the H-ion-error large, and if the end point is alkaline then the OH-ion-error will become large though the H-ion-error is small. If these were the only errors with which to reckon the best procedure would evidently be to titrate to p_H 7 where both errors are small. But this is often impossible because what we titrate is only the dissociated alkali and acid, and if at the end point we have undissociated acid or alkali left an error will arise. How much undissociated acid and alkali we have at a certain p_H depends on the dissociation constants of the substances in question.

Bjerrum gives for this error the following formula:

$$\text{relative acid-error} = 10^{p_{acid}-p_T} \times \text{the titrated amount of acid,}$$

where $p_{acid} = -\log K_{acid}$; K_{acid} being the dissociation constant of the acid and p_T the p_H of the end point.

The formula for the error due to undissociated alkali is:

$$\text{relative alkali-error} = 10^{p_{alk}-(14-p_T)} \times \text{the amount of titrated alkali,}$$

where $p_{alk} = -\log K_{alk}$; K_{alk} being the dissociation constant of the alkali.

The magnitude of these errors therefore depends on the dissociation-constant of the acid and alkali and of the p_H of the end point.

If one wants the error not to exceed 0·1 %, one must select the p_H of the end point so that it is 3 p_H-units to the alkaline side of p_{acid}, as defined above, or 3 p_H-units to the acid side of p_{alk}.

If for instance one wants to titrate acetic acid and keep this error below 0·1 % then, because the dissociation constant of acetic acid is $10^{-4.75}$, p_{acid} is 4·75, and we must titrate to an end point of p_H 4·75 + 3 = p_H 7·75. At this point the H˙-error and the OH′-error are also small, and we are therefore able to titrate acetic acid accurately with an indicator which gives an end point of p_H 7·75 or more alkaline as long as the end point does not differ so much from p_H 7 that the OH′-error is appreciable.

But if we want to titrate H_2S ($K_{H_2S} = 10^{-7}$) the p_{acid} of which is 7·00, we shall, in order to keep the relative acid-error below 0·1 %, titrate to p_H 10. For p_H 10 we get an OH′-error of $10^{-4} \times v$ cc. N solution. If the volume is 100 cc. the error is $10^{-4} \times 100 = 0{\cdot}01$ cc. N solution, and to make this error 0·1 % we must have at least 10 cc. N solution in the 100 cc. If the amount of H_2S is less than that, the titration can be performed with an accuracy of 0·1 % only if the volume is reduced.

Generally one may say that if the dissociation constant of either the acid or the alkali involved in a titration forces one to titrate at a p_H higher or lower than 7 in order to keep the relative acid-error low, then the only possible way of keeping down the OH′ and the H˙-errors is to reduce the volume; small amounts of acid or alkali cannot, therefore, be titrated exactly when using diluted solutions of low normality.

If for instance we try to titrate 10 mm.³ N acid with N/100 alkali and methyl red as indicator (end point p_H 5) in 100 cc., 10 cc. and 1 cc., then we shall get H˙-errors which are

$10^{-5} \times 101 = 0{\cdot}001$ cc. = 1 mm.³ N acid = 10 %.
$10^{-5} \times 11 = 0{\cdot}00011$ cc. = 0·11 mm.³ N acid = 1·1 %.
$10^{-5} \times 2 = 0{\cdot}00002$ cc. = 0·02 mm.³ N acid = 0·2 %.

But if we want to keep the end volume small, the amount added during the titration must be small also, and as it is important to keep the end volume nearly constant in spite of variations in the amount of fluid added during the titration, this amount must be small in comparison with the end volume. If for instance we want to titrate in 1 cc., then the amount of titration fluid must be kept below 0·1 cc. This would involve the use of a stronger titration fluid, which in itself is an advantage as in most cases it is more exact and may be kept longer. But we must then be able to add this fluid in amounts smaller than 0·1 cc. with an accuracy of fractions of 1 %. With an ordinary burette this is quite impossible, and even with Bang's microburette the accidental error of reading is easily 0·01 cc. or 10 % of what we want to add.

If we want to titrate in this way we must evidently use a very narrow burette so that 0·1 cc. can be suitably divided and read to say 0·1 mm.³. This is easily done, but it is obviously impossible to add fluid from such a burette in amounts as small as 0·1 mm.³ in the ordinary way by means

of a stopcock. Krogh [1908], in his apparatus for micro gas analysis, employed an iron screw working in mercury to move the fluid in the capillary tube of the apparatus. Using this principle it is easy to drive out from a capillary tube as small an amount of fluid as desired, but the fluid will gather at the

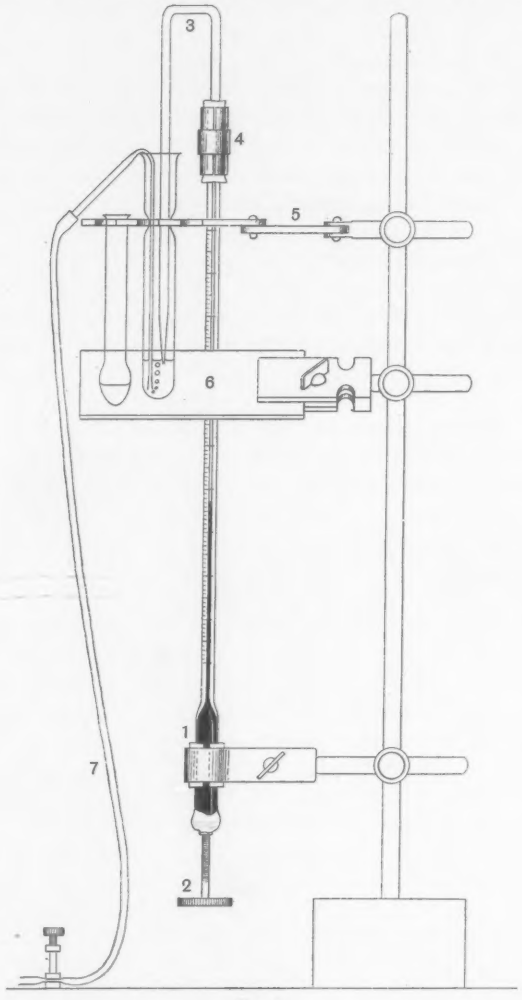

Fig. 1.

tip as a drop, and it will therefore be necessary during the titration to dip the tip of the burette into the fluid which is being titrated. As the outcome of such reasoning the apparatus to be described has been constructed.

The burette[1] consists of a capillary tube about 6–8 mm. thick with a bore of about 0·8–0·9 mm., so that the length of 0·1 cc. is between 15 and 20 cm. It is divided in mm.[3] and calibrated by measuring the length of a weighed drop of mercury at different positions in the tube. At the lower end the tube is widened (1) to take the screw (2), which is of steel and sealed in with picein or sealing wax. This part of the burette is filled with mercury, and by turning the screw the whole of the burette may be filled with mercury. The dimensions of the screw are such that one revolution corresponds to about 4 mm.[3].

As the mercury has to be below the water-solution the burette is mounted as shown in the figure. The tip is then most conveniently made as a loose part (3) and connected with the burette itself by means of a piece of rubber tubing, the connection being strengthened by means of a brass tube cut in two halves and kept together by means of a ring (4). The tip is made of a capillary tube with a bore of 0·8–1·0 mm. and holds more fluid than is used during a titration.

The burette is mounted on a stand which also carries a movable arm (5) arranged to hold the test-tubes used in the titration (two different types are shown in the figure) and a plate of white glass (6) used as background during the titration.

The arm takes three test-tubes, one of which is filled with the fluid used in the titration, one with the indicator used in a buffer solution of the p_H wanted as end point, and the third is the tube in which the titration is carried out.

If the solutions used are weaker than $N/10$ the test-tubes used should be made of a resistant glass (Jena or Pyrex).

To fill the burette for the first time with a fresh fluid the tip is removed, the mercury brought up to the top and the burette filled from a pipette by lowering the mercury, and then washed out once or twice. The rubber tube is put on the tip and this is then filled and connected with the burette, care being taken to avoid air bubbles (a single air bubble of 1–5 mm.[3] travelling forward and backward with the movement of the fluid does not interfere with the accuracy of the titration).

When the burette has been used for a titration it can be refilled by dipping the tip in the fluid and lowering the mercury.

The burette is used in the following way: on filling, the meniscus of the mercury is brought a few mm. below the zero point, the tube containing the titration fluid is removed and the tip carefully wiped with a piece of filter paper to remove all fluid adhering to the outside. Just before the titration the mercury meniscus is brought to zero and the small drop now adhering to the tip cautiously removed by touching with filter paper. All readings are made by means of a lens mounted in a holder[2], which is laid against the burette

[1] The burette with stand, as in figure, may be obtained from the workshop of the zoophysiological laboratory, Ny Vestergade 11, Copenhagen, at a price of 50 Danish kroner.

[2] As used in the micro-analysis of gases by Krogh.

in order to minimise the error due to parallax. In this way the error of the reading may be kept in the neighbourhood of 0·1 mm.³. By means of a pipette a suitable amount (0·5–5 cc.) of the fluid to be titrated is measured out in a test-tube and the indicator added.

The amount of the indicator used must, in all titrations which are to be compared, remain the same in order to keep constant the error due to participation of the indicator in the reaction. This error is under these circumstances greater than in an ordinary titration, because the amount of indicator needed to give a sharp end point in such a small volume is comparatively large. For this reason the indicator is best added as 0·1 cc. of a very dilute solution, or a suitable amount of indicator is once for all added to the stock solution.

The tube is now placed in the slot of the arm and raised until the tip of the burette just dips into the fluid. During the titration the fluid is mixed by means of a stream of small air bubbles from a very fine glass tube dipping into the fluid and connected with air pressure (7). The air is filtered through a tube filled with soda-lime to remove carbon dioxide. If air pressure is not available the small amount of air needed may easily be supplied from two 5-litre bottles half filled with weak NaOH-solution and connected to form a gasometer. The bubbling is regulated by means of a clip. By turning the screw of the burette the titration fluid is now added in small amounts till the end point is reached, and the burette is then read again.

To show the accuracy of the method I append a few examples.

By means of a microburette from 5 to 100 mm.³ $N/200$ H_2SO_4 were measured into titration tubes. 1 cc. H_2O and 0·1 cc. of a 0·001 % solution of methyl red were added and the tubes titrated with $N/200$ NaOH. The volume in these titrations was at the end between 1·1 and 1·3 cc. If we assume that we are able to hit the p_H wanted (5·5) within 0·5 p_H, we shall at the end of the titration have a p_H varying between p_H 5·25 and p_H 5·75. These two values give the following results for the H˙-error:

$10^{-5.25} \times 1{\cdot}2$ cc. N $H_2SO_4 = 0{\cdot}0067$ mm.³ N $H_2SO_4 = 1{\cdot}35$ mm.³ $N/200$ H_2SO_4

and

$10^{-5.75} \times 1{\cdot}2$ cc. N $H_2SO_4 = 0{\cdot}0021$ mm.³ N $H_2SO_4 = 0{\cdot}4$ mm.³ $N/200$ H_2SO_4.

The accidental error in judging the end point will therefore give rise to variations in the result of $\pm$ 0·5 mm.³.

Moreover, we have in each of these titrations four different readings of the burettes on each of which there may be an error of 0·1 mm.³, so that we must expect a maximum error of $\pm$ 0·9 mm.³. The mean error will of course be smaller.

The following table shows that two titrations of the same quantity of $N/200$ H_2SO_4 may differ from 0 to 1·3 mm.³.

The table shows, as might be expected, that the results have a systematic error; the quantity of NaOH used is less than the theoretical. This is partly due to the fact that the alkali is not exactly $N/200$ and partly to the H˙-error

mm.³ $N/200$ acid measured	mm.³ $N/200$ NaOH used in titrations		Difference between the two titrations	mm.³ NaOH + 1·55 / 0·954	
5	2·5	3·5	1·0	4·05	5·05
10	8·0	8·5	0·5	10·00	10·50
20	17·0	17·4	0·4	19·40	19·90
30	26·8	26·9	0·1	29·75	29·85
40	35·9	36·8	0·9	39·30	40·15
50	46·2	46·9	0·7	50·00	50·75
60	56·0	56·1	0·1	60·40	60·50
70	65·5	65·7	0·2	70·35	70·50
80	73·9	75·2	1·3	79·00	80·50
90	84·5	85·1	0·6	90·20	90·80
100	93·1	94·3	1·2	99·20	100·40

and the participation of the indicator in the reaction. If we take these errors into consideration we get the following formula:

$$\text{mm.}^3 \text{ acid} = \frac{\text{mm.}^3 \text{ NaOH} + y}{x}$$

and from the results in the table it is possible to find the values of x and y by the method of least squares.

They are:

$$x = 0{\cdot}954 \text{ and } y = 1{\cdot}55,$$

which means that 100 mm.³ acid is equal to 95·4 mm.³ NaOH and that the results obtained by the titrations are moreover 1·55 mm.³ too low on account of the indicator and $H^{\cdot}$-errors.

In the last two columns of the table are given the values of the titrations corrected in this way. The mean error in a single example of these corrected titrations is 0·52 mm.³ $N/200$ solution, which corresponds, if used for instance in an ammonia determination, to 0·000036 mg. N.

In daily use it is of course inconvenient to find the titre of the alkali with respect to the acid by means of least squares. It is best ascertained by an ordinary macrotitration. With stronger solutions the error is negligible, so that the microtitration is sufficient.

If we try to titrate to a point closer to p_H 7 we shall, as has been said, have a smaller $H^{\cdot}$-error. In a series of titrations of $N/200$ acid in a volume of 1 cc. to p_H 6·25 using bromocresol purple as indicator I found a mean error in a single determination of 0·27 mm.³ $N/200$ H_2SO_4 or 0·000019 mg. ammonia N.

Using stronger solutions still better results are obtained: working with methyl red and 0·1 N solutions, and titrating in 1 cc., I found a mean error of 0·2 mm.³ and in 5 cc. of 0·3 mm.³.

This accuracy is sufficient for most purposes, but if still greater accuracy is wanted it will be quite possible to get a little further. The burette may easily be made to measure out still smaller fractions of 1 mm.³—the difficulty will be to observe the change in colour in the small volume required in such cases, but I think that it will be possible to titrate in a volume of about 0·1 cc. which would increase the accuracy to a considerable extent.

As examples of the practical use of the apparatus some methods for the determination of several blood constituents will be published shortly.

SUMMARY.

1. In a brief discussion of the theory of titration the influence of total volume is considered, and it is shown that many titrations can be performed with advantage as microtitrations.

2. An apparatus for microtitration and its use is described. The apparatus allows the addition of fluid from a burette in accurately measured quantities down to 0·1 mm.^{3}.

I wish to express my sincere gratitude to Prof. Krogh for his kind interest and helpful advice during the work.

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XLII. THE DETERMINATION OF UREA IN 0·1 cc. OF BLOOD BY MICROTITRATION.

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Both in the laboratory and in the clinic determinations of blood-urea at short intervals of time are often required, and it is difficult to make them because of the amount of blood needed.

By means of the method of microtitration described in the preceding paper it is possible to overcome this difficulty.

The method to be described is a modification of the Van Slyke method, depending on conversion of the urea into ammonia by means of urease and subsequent aeration of the ammonia into acid and titration of the excess of acid.

The following stock reagents are needed: $N/30$ solutions of H_2SO_4 and NaOH (and if uraemic blood is to be expected $N/10$ solutions); a 0·005 % solution of methyl red[1]; octyl alcohol; saturated K_2CO_3 solution; a 10 % solution of a urease preparation (freshly prepared each day) in water, or, better, in a buffer solution at p_H 7 (Sørensen phosphate buffer p_H 7 diluted 20 times)[2].

The determination is carried out in the following way:

0·1 cc. of blood is measured out in a test-tube in which have previously been placed 0·5 cc. of water and a trace of powdered sodium oxalate. The pipette is rinsed twice with the water and 0·1 cc. of the urease solution is added. The tube is closed, and after the lapse of the usual time (10 mins. at 40°, 30 mins. or more at room temperature) 0·5–1 cc. of saturated K_2CO_3 and a drop of octyl alcohol are added, and the tube is now ready for aeration.

The titration tubes, about 12 × 1·6 cm., which must be flat-bottomed and of resistant glass, are in the meantime prepared as follows: 0·1 cc. $N/30$ H_2SO_4 is measured from a microburette into a titration tube, 4 cc. of water (best redistilled from acidified distilled water), a drop of octyl alcohol, and 0·1 cc. of the indicator solution are added. If a microburette is not used 1 cc. of $N/300$ H_2SO_4 (diluted fresh every day from the $N/30$ solution with

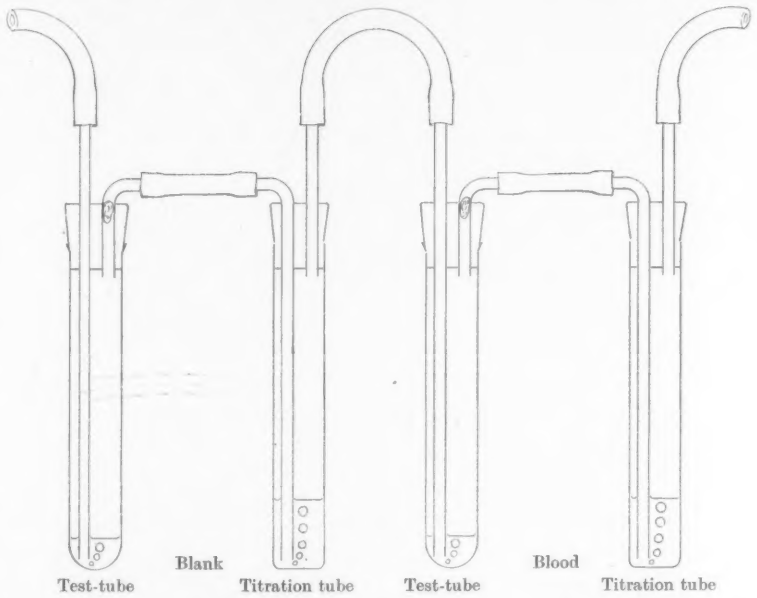
[1] Instead of methyl red a 0·01 % bromocresol purple solution may conveniently be used as indicator—it gives a sharper end point, but it involves the necessity of using solutions absolutely free from carbon dioxide.

[2] 3·9 cc. of a 0·9078 % KH_2PO_4 solution + 6·1 cc. of a 1·1876 % Na_2HPO_4 solution diluted to 200 cc.

a weak indicator solution) is measured out with an accurate pipette and 3 cc. water and octyl alcohol are added. The test-tubes are then connected as shown in the figure, a loose plug of cotton-wool being placed in the tubes between test-tubes and titration tubes, and a current of air, freed from ammonia and carbon dioxide by means of H_2SO_4 and soda-lime, is passed through them.

The time required for aeration depends of course on the rate of the air current, but I have found it possible to drive over all ammonia in 30–45 mins. One or two blank experiments must be run at the same time.

After disconnection the contents of the titration tubes are titrated by means of the microburette with $N/30$ NaOH, as described in the preceding



paper. The difference between a blank and a blood-tube multiplied by the titre factor indicates how many mg. urea are contained in 100 cc. of blood.

Example: 100 mm.³ H_2SO_4 = 97·3 mm.³ NaOH

$$\text{Titre factor} = \frac{100}{97{\cdot}3} = 1{\cdot}028.$$

Blank = 95·1
Blood = 69·7
25·4 × 1·028 = 26·1 mg. urea per 100 cc. blood.

This method has in my hands given a mean error of ± 0·7 mg. urea on a single determination, but since it is easy to obtain duplicate analyses it allows of a determination with an accuracy of about 0·5 mg.

As examples I shall give the following experiments:

1. Urea solutions of known concentration:

Urea, mg. per 100 cc.	Found
28·95	29·4
	28·5
	28·2
115·8	115·5
144·75	144·9

2. Blood urea:

	Time	mg. per 100 cc.
Fasting	9.30	21·2
		21·2
	10.00	21·0
		21·3
2 eggs eaten	10.15	
	10.50	21·5
		21·0
	11.25	21·5
		21·2
	12.10	25·8
		25·3

3. Blood urea:

	Time	mg. per 100 cc.
	1.45	18·8
		19·7
20 g. urea taken in water	1·52	
	2.10	36·8
		37·3
	2·42	46·2
		47·6
	3·25	56·0
		56·3

XLIII. THE SURFACE TENSION OF GELATIN SOLUTIONS.

By JAMES HALLEWELL ST JOHNSTON and GEORGE THOMAS PEARD.

From the laboratory of Messrs Bass, Ratcliff and Gretton, Ltd., Burton-on-Trent.

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In the course of some experiments on a vegetable protein, a marked variation of the surface tension of its solution with changing hydrion concentration was observed. Reference to the literature showed that beyond the well-known surface tension lowering effect of proteins and other colloids, the few observations that have been made have usually taken no account of the hydrion concentration.

It was therefore decided to investigate the effect of the p_H on the surface tension of gelatin solutions, in order to see if the surface tension varied in a similar manner to the other physical properties with change of p_H. Gelatin was used as it can be easily obtained in a relatively pure state and its other physical properties are well known.

Reynolds [1921] gave the value 71·1 dynes/cm. for a fresh 0·5 % gelatin solution as determined by the capillary rise method; this fell to 65·5 dynes/cm. on standing 24 hours, which he attributed to increased surface concentration; he also determined the interfacial tension of gelatin solution against benzene by the drop method.

Mardles [1924] determined the surface tension of gelatin in water and other solvents at various temperatures. The only investigation taking account of the p_H that we can find is a recent paper by Davis, Salisbury and Harvey [1924], who studied various commercial gelatins and gelatin rendered ash-free by Loeb's method, using the drop number method of determining surface tension. Different curves were obtained for various gelatins, the most marked variations with p_H being shown with the ash-free gelatin. This gave a maximum surface tension at about p_H 3·4. They also studied the effects of concentration, time and temperature at constant p_H. No explanation was advanced by them of the changes observed.

The surface tension between the particle and the medium, as an important factor in the stability of colloids, is considered in the theories of Bredig [1901], Donnan [1901], Freundlich [1922], von Weimarn [1911] and others; further references to the literature are given in the *Fifth Report on Colloid Chemistry* [1923].

At the isoelectric point the charge on the particle is a minimum, hence,

according to the Lippman effect, the surface tension should be a maximum. Burton and Wiegand [1912] have, however, shown that an electrical charge does not affect the surface tension *per se*, but in general acts so as to oppose the effect of the surface tension.

Loeb [1922] has shown that Donnan's theory of membrane equilibrium explains the colloidal properties of gelatin, and in particular the membrane potentials. Thus we should expect to find some relationship between the P.D.-p_H curves and the surface tension-p_H curves for gelatin.

Experimental.

The measurement of the surface tension was made by Sugden's maximum bubble pressure method [1922, 1924]. This was found to be convenient, rapid and reasonably accurate, besides being unaffected by vibration.

Reynolds [1921] has shown that a dynamic method is desirable for colloids, otherwise the surface tension changes with age owing to increasing concentration in the surface layer; hence the surface tension by a static method falls on standing.

The apparatus was calibrated with pure benzene and was accurate to within 0·3 % for pure liquids, but probably less accurate for colloidal solutions.

When not in use the apparatus was kept filled with either nitric or chromic acid; it was washed with tap water and distilled water before use, and between each determination it was washed with warm nitric acid, and subsequently with tap and distilled water. Similarly all vessels used were pickled in chromic acid, then washed with tap and distilled water and drained dry. These precautions were necessary to ensure freedom from grease.

The gelatin used was Nelson's Gold Label powdered, of an original ash content of 1·5 % and p_H about 5–6. To render it ash-free a modification of Loeb's method of washing was adopted as being more convenient. 15 g. of powdered gelatin were soaked for 24 hours with one change in 500 cc. of a sodium acetate-acetic acid buffer mixture of p_H 4·7 and concentration $N/50$, then for a further 24 hours with distilled water with one change. The temperature was kept below 5° during the soaking. The gelatin was finally washed by decantation and at the pump on a Buchner funnel with 2–3 litres of distilled water, keeping the temperature as low as possible, usually below 8°. The washed gelatin was dissolved in about 300 cc. of distilled water by immersing the beaker in a water-bath kept at 52° for half-an-hour exactly, in which time the gelatin completely dissolved and the temperature of its solution rose to 50°. The warm solution was filtered to remove any undissolved particles, and the concentration was determined by evaporating a portion in a platinum dish in a steam oven for 12 hours by which time the weight had become constant. The dried gelatin in the dish was ashed. The ash content by this method was found to be between 0·08 and 0·13 % on the dry gelatin, which is equivalent to a salt concentration of about $M/30{,}000$, calculating as $Ca_3(PO_4)_2$, in a 1 % solution of gelatin..

The gelatin solution was just remelted in the 52° water-bath and diluted to make the 2 % stock solution, about 500 cc. being obtained. This also set to a gel and each time before use it was just melted in the water-bath, then rapidly cooled to 25° and the required amount withdrawn in a pipette calibrated for that temperature. 15 cc. of the 2 % gelatin solution were added to 15 cc. of solution containing the requisite amount of $N/10$ acid or alkali and distilled water, contained in a conical flask fitted with a rubber bung washed free of grease with spirit. A drop of toluene was added as a preservative, as this was found to have no effect on the surface tension. The solutions were kept for 24 hours in an oven at 25° before determination, in order to allow complete equilibrium to be established. The surface tension was then determined at 25°. In the case of the determinations at 40°, the solutions were only kept an hour at that temperature before determination.

In view of the effect of pretreatment of the gelatin on its physical properties, the above routine was strictly adhered to, although it was later found that the surface tension does not vary with time to an appreciable extent once equilibrium has been established, except in the case of isoelectric gelatin.

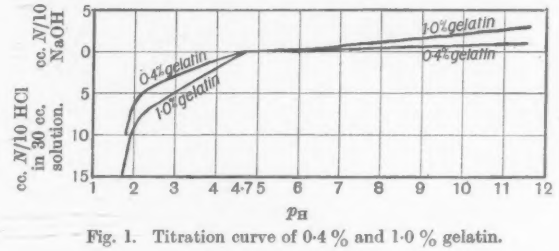

Fig. 1. Titration curve of 0·4 % and 1·0 % gelatin.

THE EFFECT OF HYDROGEN-ION CONCENTRATION.

The hydrogen-ion concentrations were determined colorimetrically, using Walpole and Sörensen's standards and a Cole and Onslow's comparator, and were accurate to 0·1 p_H. The titration curve, cc. $N/10$ acid or alkali in 30 cc. against p_H, was plotted as in Fig. 1. The results above p_H 7·5 were only approximate.

In the determination of the surface tension by Sugden's bubbler, small jets giving a pressure difference of between 7 and 13 cm. alcohol were used, and the bubble rate was usually one bubble per 2–3 seconds on the small jet. The mean of 5 or 6 readings at various bubble rates of about that order was taken as the required value. According to Sugden too slow a rate would give lower values as well as allowing minor leaks in the apparatus to take effect. Too fast a rate (greater than 1 per second) led to excessive frothing and too high values. The determination of the surface tension of the isoelectric gelatin was found the most troublesome. As will be seen from the time curves in Fig. 4, 1 % gelatin p_H 4·7 gradually flocculated in a varying time with different samples, no doubt owing to different ash content or absorbed CO_2 or NH_3

from the air; the surface tension therefore was not determined within 24 hours but after flocculation had occurred, usually in 36–48 hours, though sometimes longer. Owing to the presence of solid particles, a permanent froth was formed which added to the difficulty of determination, and also the values fell on prolonged bubbling, again possibly owing to absorption of CO_2 or NH_3 from the air passing through. From the sharpness of the curves at p_H 4·7 in Fig. 2 this fall of surface tension is easily understood.

The results for 1 % gelatin in presence of HCl, H_2SO_4 or NaOH at 25° and for gelatin in presence of HCl or NaOH at 40° are shown in Fig. 2, all showing a uniform sharp variation of surface tension with p_H, particularly on the acid side.

In view of recent work by Knaggs, Manning and Schryver [1923] on the further purification of gelatin by electrolysis and recrystallisation, a sample of gelatin of ash content 0·08 % was electrolysed according to their method.

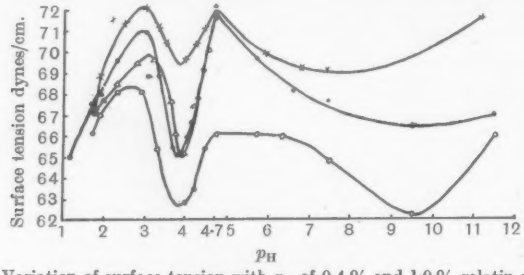

Fig. 2. Variation of surface tension with p_H of 0·4 % and 1·0 % gelatin solutions at 25° and 40°.

× 0·4 % gelatin in presence of HCl or NaOH at 25°
● 1·0 % " " " " 25°
○ 1·0 % " " " " 40°
△ 1·0 % " " H_2SO_4 at 25°

We are indebted to Mr R. W. W. Sanderson, of the General Electric Co., for carrying out this electrolysis. The resulting gel was ash-free and an attempt was made to recrystallise it from a 1 % solution, but possibly owing to absorption of CO_2 or NH_3 from the air, it did not flocculate completely and the recrystallised gelatin was difficult to separate either by centrifuging or filtering. The separated gelatin was redissolved, giving a 0·8 % stock solution. The surface tension of the resulting 0·4 % gelatin in presence of HCl or NaOH at 25° is also shown in Fig. 2.

As electrical facilities were not available on the spot, it was not possible to flocculate electrically according to the latest method of Knaggs and Schryver [1924].

The effect of Neutral Salts and Non-Electrolytes.

As typical salts of uni- and bi-valent acids $NaNO_3$ and Na_2SO_4 were used, and as a typical non-electrolyte glucose (99 % pure). These determinations were carried out at p_H 3·3, and the solutions were prepared either by directly

weighing out the required amount of salt or sugar and dissolving in 4 cc. $N/10$ HCl + 11 cc. distilled water, or else by making up a solution and diluting so that 11 cc. of it together with 4 cc. HCl gave the final required concentration in 30 cc. The surface tension at p_H 3·3 with no additions was the mean of three separate determinations. The p_H of the solutions was found to fall gradually from p_H 3·3 to p_H 3·15 with increasing salt concentration up to $M/16$.

As will be seen from Fig. 3 the surface tension falls with increasing salt concentration and more for Na_2SO_4 than for $NaNO_3$. The effect of the fall of p_H would tend to raise the surface tension, as can be seen by consideration of the curve for gelatin in HCl in Fig. 2. The non-electrolyte glucose had very little effect even in concentration $M/4$—the slight lowering being probably due to the ash content of the glucose.

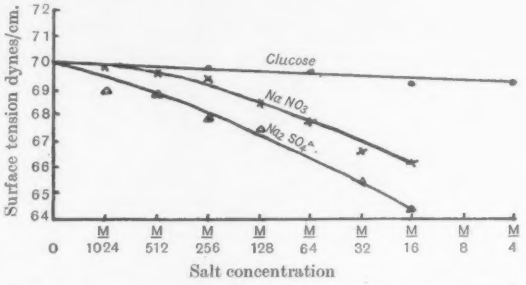

Fig. 3. Effect of neutral salts and of glucose on surface tension of 1 % gelatin in presence of HCl at p_H 3·3 and 25°.

The Effect of Time.

Although the results in Figs. 2 and 3 were all obtained under uniform conditions, the effect of time on the surface tension was studied in one or two cases. The determinations were started immediately the gelatin had been dissolved and filtered.

Equilibrium was apparently reached at p_H 3·3 in an hour or so, and then remained constant within experimental limits for the several days over which it was observed. With isoelectric gelatin, the surface tension rose gradually from a low value of about 66 dynes/cm. up to 71·5 in 72 hours, *i.e.* approaching that of water, as flocculation proceeded. In other cases where no previous readings of the surface tension were taken it had risen very nearly to 72 in 36–48 hours, when complete flocculation had occurred. The results are shown in Fig. 4.

Discussion of Results.

Considering first the surface tension-p_H curves at 25° in Fig. 2, it will be seen that the effect of decreasing the concentration of gelatin from 1 % to 0·4 % is to increase the surface tension as would be expected. All the curves at 25° show a maximum at p_H 4·7, as was expected, values approaching that of water being obtained. This corresponds to a very low P.D. and solubility

and a maximum of neutral un-ionised particles at the isoelectric point, but presumably there is some dissociation there, causing a very slight lowering of surface tension. The curve at 40° differs from those at 25° in that the surface tension at the isoelectric point is considerably lower than that of water at 40° and that there is no sharp maximum. There is evidently considerably more gelatin in solution at that temperature, and presumably more dissociation into electrically equal amounts of positive and negative ions. Pauli [1921] has shown from mathematical considerations that this would be expected.

The 40° curve shows a marked flattening between p_H 4·6 and 6·0, probably due to the above increased dissociation; the dissociation constants of positive and negative dissociation may increase at different rates with temperature rise, and from the expression $C_H = \sqrt{\frac{K_a K_w}{K_b}}$ for the hydrion concentration of the isoelectric point, where K_a, K_b and K_w are the dissociation constants of positive and negative dissociation of the protein and of water respectively,

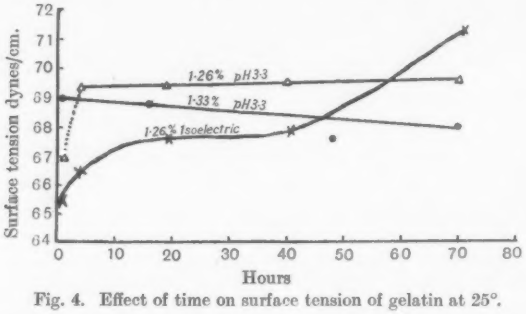

Fig. 4. Effect of time on surface tension of gelatin at 25°.

we should expect a small change in the p_H of the isoelectric point with temperature. Our results are not accurate enough to determine this, beyond the marked flattening, except that, whereas at 25° the surface tension of isoelectric gelatin was found to be practically independent of concentration, as is to be expected theoretically from the calculations of Pauli, Michaelis and Sörensen, at 40° the surface tension at p_H 4·7 was found to vary with the concentration values:

1 % gelatin, surface tension = 66·1 dynes/cm.
2 ,, ,, ,, ,, = 64·4 ,,
6 ,, ,, ,, ,, = 62·3 ,,

This may be an indication that the isoelectric point at 40° is not exactly at p_H 4·7.

All the curves for gelatin in presence of HCl show a sharp point of minimum surface tension at p_H 3·8, and for gelatin in presence of H_2SO_4 at p_H 3·9–4·0. This agrees well with Loeb's calculation and determination of the maximum P.D. of 1 % gelatin at p_H 3·8–4·0. The P.D. of gelatin in presence of H_2SO_4 is only two-thirds that of gelatin in presence of HCl, though no difference in the

lowering of surface tension was found by us, and, as will be seen later from the effect of neutral salts, the surface tension lowering cannot be considered as entirely dependent on the P.D. Loeb further shows that the P.D. decreases steadily from this maximum value at p_H 3·8–4·0 down to p_H 1. We, however, find a well-marked maximum value again for the surface tension at about p_H 3·0, after which the surface tension again falls rapidly with increased acid concentration. Davis, Salisbury and Harvey show a similar maximum about p_H 3·4. This may be due to a change in the gelatin molecule with formation of "acid protein." This conclusion is supported by the titration curves shown in Fig. 1, which agree with those of other workers in showing a sharp change of direction about p_H 2·5, indicating increased combination of acid. Henderson, in Osborne's *The Vegetable Proteins* (1923), shows that at p_H 1–2, and again at p_H above 10, the titration curves of proteins no longer agree with those required by theory and attributes this to changes in the molecule in more concentrated acid or alkali. Pauli finds that other proteins with strong acids are most sensitive to coagulating agents such as alcohol at a p_H of about 3·0. According to Bredig, Burton [1923] and others, a high surface tension would be conducive to precipitation. Surface tension may show this change in the molecule before the titration curves do, as it is obviously very sensitive to small changes; hence our maximum occurs earlier, about 2·8–3·0. On the other hand, the drop in surface tension after this maximum may be due to increased dispersion brought about by peptisation. Further experiments are necessary to find out what is occurring in this region.

On the alkaline side a minimum surface tension is observed at a p_H of about 9–10, which agrees with Loeb's observed and calculated values for maximum P.D. and also for swelling of gelatin in NaOH solutions.

The results obtained by the addition of neutral salts to gelatin in presence of HCl are entirely unexpected. The Donnan theory of membrane equilibrium demands that such addition should depress the P.D. of the gelatin and the more so the greater the concentration of added salt. Moreover, the depression of the P.D. caused by a salt of a univalent acid, such as sodium nitrate, should be only two-thirds that produced by equimolar amounts of a salt of a bivalent acid, such as sodium sulphate. If surface tension is influenced by the P.D., depression of the latter should lead to a rise in surface tension. We find, however, as will be seen from Fig. 3, that the addition of a neutral salt produces a depression of the surface tension very nearly proportional to the concentration of the added salt. Curiously enough the depression produced by a salt of a univalent acid is approximately two-thirds of that produced by a salt of a bivalent acid when the concentration of the added salt exceeds $M/256$. Berczeller [1917] found that the addition of salts lowered the surface tension of protein solutions. Davis, Salisbury and Harvey similarly found that the surface tension of ash-free gelatin was much higher than that of a commercial gelatin containing mineral impurities.

The addition of a non-electrolyte, such as glucose, does not alter the

surface tension of the sol except for a slight depression due to the mineral impurities of the glucose. Since it can play no part in the Donnan equilibrium, this is to be expected.

Recent work by Manning [1924] on electrically flocculated isoelectric gelatin has shown that this may be peptised or dispersed to a stable gel by very small concentrations of acid and alkali, whilst relatively larger concentrations of neutral salts will produce the same effect. Evidently the addition of neutral salts tends to increase dispersion of the gelatin, making relatively more gelatin in the sol form. This affects the surface tension, causing a lowering.

The surface tension-time curves in Fig. 4 show that the rapidly cooled gelatin solution has a low surface tension, which at p_H 3·3 rises within an hour or so to its equilibrium value, which it then maintains within experimental limits over the period observed. Gelatin at the isoelectric point similarly starts at a low value, which rises gradually as the solution changes over to the gel form and flocculates, when a maximum surface tension near that of water is reached. Smith [1919] has shown by following the mutarotation that there is an equilibrium between the sol and gel forms of gelatin between the temperatures of 15° and 35° depending on the temperature.

Evidently then anything which increases the amount of the sol form in the solution, whether it be rise of temperature, presence of acid or alkali, or neutral salts, will cause an increased lowering of surface tension. Donnan's membrane equilibrium theory apparently accounts quantitatively for the ionisation of ash-free gelatin and for the membrane potentials observed by Loeb, but as far as surface tension is concerned, the other factors, such as peptisation of the neutral gelatin particles by neutral salts, and strong acids in higher concentration, and the physical state of the gelatin as influenced by temperature and time must also be taken into account. Further, the effect of strong acids at concentrations giving a p_H less than 3 is more marked on the surface tension than on any other property, though as to what actually occurs further investigation is required. It will be seen then that it is not possible in our present state of knowledge of the relationship of the surface tension of solutions to the many factors affecting it, to make any generalisations, except that lowering of surface tension is coincident with increased dispersion and stability of the colloid, and that a high surface tension tends to coagulation. As a means of determining the isoelectric points of proteins whose other properties are difficult of determination, the method may be useful, provided distinction is possible between the true isoelectric point and the maximum produced at a higher acid concentration.

Further work with other proteins is being undertaken in this direction.

SUMMARY.

1. No direct quantitative relationship could be traced between the surface tension of a colloidal solution and the potential difference between the particles and the medium.

2. The surface tension of gelatin solutions is found to be a maximum at the isoelectric point (p_H 4·7); a minimum at p_H 3·8–4·0 on the acid side, and in the neighbourhood of p_H 9 on the alkaline side. A second maximum is found at p_H 2·8–3·0, after which the surface tension decreases steadily. It is suggested that this second maximum marks the point at which the formation of "acid-gelatin" begins.

3. Increase of temperature and addition of electrolytes both cause a lowering of the surface tension of gelatin solutions. This indicates that increased solubility or peptisation of the gelatin lowers the surface tension.

4. The surface tension of gelatin solutions is little affected by time after equilibrium is attained, which occurs in a few hours at 25°, except in the case of isoelectric gelatin, whose surface tension slowly rises nearly to that of water as flocculation proceeds.

We are indebted to Dr A. L. Stern and Dr A. Slator for advice during the course of this investigation, and to the Directors of Bass, Ratcliff and Gretton, Ltd., for permission to publish our results.

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XLIV. THE OCCURRENCE OF DIASTASE IN THE CEREBRO-SPINAL FLUID.

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(*Received February 25th, 1925.*)

THE earliest investigations into the chemistry of cerebro-spinal fluid were carried out on fluid obtained from cases of hydrocephalus and spina bifida, and so we find that as early as 1891 Toison and Lenoble [1891] reported that they found the cerebro-spinal fluid of a hydrocephalic to have a slight amylolytic power. Several years later Cavazzani [1896] observed similar results in two such cases. Galletta [1908], who examined eleven normal cases, only found amylase in two of them. More recently Mestrezat [1912] stated that in two normal fluids he found just a trace of diastase, and in his book he comes to the conclusion that normal cerebro-spinal fluid has no amylolytic power or only very slight traces of it. Stocks, however, has stated [1916] that he had succeeded in demonstrating amylase in every fluid of the body which he had examined, including the cerebro-spinal fluid.

It is important to note that the term "normal" as applied to cerebro-spinal fluid must be considered merely relative, for the mere fact of there existing a reason for withdrawing some of the fluid from the living person makes the case a pathological one. Again, post-mortem fluid is known to differ from that in the living, so that observation on post-mortem cerebro-spinal fluid cannot be considered normal. It is only by considering a series of fluids taken during life and post-mortem that it is possible to arrive at some conclusion as to what the normal is likely to be. Both Galletta and Mestrezat (*vide supra*) define their "liquides normaux" as obtained from "sujets chirurgicaux."

The cases in the investigation about to be described fall into two groups. Firstly those in which the cerebro-spinal fluid was taken during life and secondly those in which the fluid was taken at known intervals after death. In many of the cases in the latter group a specimen of blood was taken at the same time for purposes of comparison.

Method. The method used for estimating the diastatic content of the cerebro-spinal fluid was a slight modification of the method for other body fluids, described in conjunction with Dodds [1924].

The following solutions are required:

1. Buffer solution. This is made by mixing 15 cc. of Sörensen's phosphate solution A with 85 cc. of solution B. The resulting solution should have a p_H of 6·1. Solution A: 11·876 g. of $Na_2HPO_42H_2O$ dissolved in 1 litre of boiled

distilled water. Solution B: 9·078 g. of KH_2PO_4 dissolved in 1 litre of boiled distilled water. The solutions are kept in paraffin-coated bottles, and a drop of toluene is added to prevent the growth of moulds.

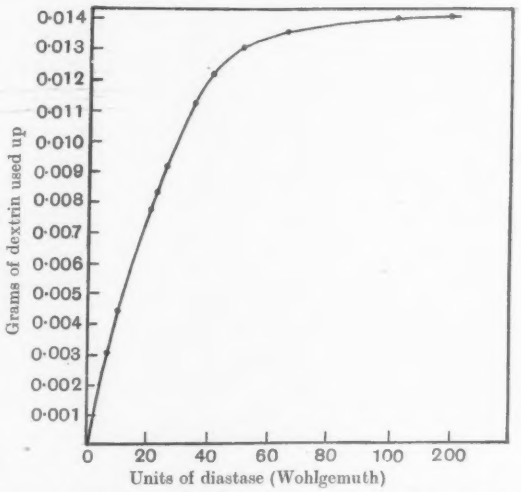
2. Standard dextrin solution: 0·75 g. of pure erythro-dextrin is dissolved in a small volume of boiled distilled water, and the volume is made up to 100 cc.

3. Iodine solution:

$N/10$ iodine in potassium iodide solution	...	20 cc.
Saturated ammonium sulphate solution	...	500 ,,
Distilled water		1000 ,,

Procedure. 1·5 cc. of cerebro-spinal fluid are pipetted into a tube and 6 cc. of buffer solution are added. The mixture is well shaken. 0·5 cc. of this buffered mixture is pipetted into a test-tube, graduated at 30 cc., and 1·0 cc. of the standard dextrin solution added. After thorough shaking the tube is incubated in a water-bath at 37° for half-an-hour. Whilst the incubation is proceeding the standard is prepared. This is made up by pipetting 1·0 cc. of standard dextrin into a tube graduated at 30 cc.

When the incubation period is complete the tube is removed from the water-bath, and 15 cc. of the iodine solution are added to it and to the standard. The volume of each is then made up to the mark, mixed and compared in the colorimeter, the standard being set at 20 mm.



Small-scale curve to show relation between units of diastase and grams of dextrin used up.

The amount of dextrin present in the unknown is calculated by the familiar colorimetric ratio—that is: $\frac{20}{X} \times Y$; where X is depth in millimetres of unknown, and Y is the quantity of dextrin in the standard. Since 1 cc. of 0·75 % dextrin

solution—that is, 0·0075 g. of dextrin—was present before incubation, the amount of dextrin used up can be calculated by subtracting from this the figure obtained in the above calculation. By reference to the curve this can be converted into Wohlgemuth units.

Group I. The cerebro-spinal fluid in this group was taken during life. Twenty-eight cases were examined with the following conditions:

Tabes	6
Syphilis (primary and secondary)	5
Uraemia	3
General paralysis of the insane	2
Tuberculous meningitis	2
Cerebral tumour	2
Cerebral syphilis	2
Encephalitis lethargica	1
Neuritis	1
Meningo-myelitis	1
Acute anterior polio-myelitis	1
Cerebro-spinal meningitis	1
Infective endocarditis	1

In addition, three samples of cerebro-spinal fluid were examined which were obtained by collecting the fluid drawn off before the injection of a spinal anaesthetic.

In the uraemic cases the urea content of the fluids was estimated for diagnostic purposes. These figures will be referred to later.

Results. In all the thirty-one cases except two not a trace of diastatic enzyme was detected. In one case of tabes and one of secondary syphilis only a trace (less than one unit) of diastase was found.

Group II. In the thirty-six cases comprising this group the spinal fluid was collected at known intervals post-mortem. Blood was taken at the same time. Urea estimations were carried out on the specimens to show the presence, if any, of nitrogen retention, for it is reasonable to expect to find in cases showing a high urea content accumulation of other substances, including perhaps the enzyme diastase.

Results. Only seven of these cases were observed to contain diastase in the cerebro-spinal fluid. From the table given below it will be seen that four of the seven show marked urea retention:

		Blood		Cerebro-spinal fluid	
Case	Hours after death	Urea mg. per 100 cc.	Diastase units	Urea mg. per 100 cc.	Diastase units
1	16	284	8	261	8
2	7	90	7	82	2
3	24	44	5	37	3
4	8	104	4	91	2
5	11	101	30	91	3
6	21	28	3	26	4
7	10	—	4	—	1

That the cause of the urea retention is not associated with the appearance of the diastase in the cerebro-spinal fluid will be seen when it is stated that the urea figures for the uraemia cases in Group I were 101, 102 and 143 mg. per 100 cc. No trace of diastase was found in these latter cases where the fluid was withdrawn during life.

Discussion.

It appears that during life, whatever the condition is, it is rare to find diastase in the cerebro-spinal fluid. It will be admitted that the nearest approach to normal fluid was obtained from cases punctured for spinal anaesthesia, and it is very significant that none of these contained any diastase. Moreover, twenty-six of the cerebro-spinal fluids derived from ante-mortem sources yielded similar results, and only two were found to contain any diastase at all, this being under one unit. It is very unlikely, therefore, that diastase is a normal constituent of cerebro-spinal fluid. In all the cases taken within 24 hours after death about 20 % were found to contain appreciable quantities of diastase. The presence of the enzyme was not observed in any case until about 7 hours after death, quite independently of the occurrence of nitrogen retention. It is therefore a post-mortem effect.

Summary.

1. The presence of diastase in the cerebro-spinal fluid of human beings during life is extremely rare.

2. Approximately 7 hours after death roughly 20 % of cerebro-spinal fluids were found to contain definite amounts of the enzyme.

3. The appearance of diastase in the cerebro-spinal fluid is a post-mortem effect occurring after about 7 hours.

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XLV. THE ESTIMATION OF AMMONIA AND UREA IN BLOOD AND URINE.

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(*Received January 16th, 1925.*)

The principles underlying the chemical changes involved in the method here described are exactly those of the well-known Folin macro aeration method. But the objections to the latter are that the apparatus is very cumbersome, and unless extremely well set up is likely to give too low results.

The apparatus I here propose is that designed by Cannan and Sulzer [1924] for the estimation of alcohol in blood. The great advantage of the apparatus is that it is very compact and easy to handle. The estimation takes less time than in the ordinary aeration apparatus; the loss of ammonia and errors due to rubber connections are cut down to a minimum. This form of apparatus can be quite easily used by students in class. The great advantage in time and ease with which the whole operation is carried out makes it desirable that the details of the procedure should be described.

Method for Ammonia in Urine.

In the outer vessel (Fig. 1) place 20 cc. urine. To the inner vessel add 20 cc. $N/10$ H_2SO_4, a few drops of methyl red and enough distilled water to cover the end of the spiral. Insert the inner part in position, then make connections at X with a sulphuric acid washer and at Y with a pump. Raise the cork and deliver into the bottom of the jacket 5 g. anhydrous Na_2CO_3 from a narrow test-tube. Press the cork in tightly, place the apparatus in a bath at 40° and aerate with a very brisk current of air for 20 to 30 minutes. After this time disconnect from the pump and washer, remove the inner vessel and wash this well on the outside. Then, holding the apparatus over a funnel placed in a flask, remove the inner part, transfer the contents to the flask and wash all the parts very thoroughly. Then titrate the contents of this flask with $N/20$ NaOH. The accuracy of the method has been tested by using a standard ammonium sulphate solution (1 cc. = 1 mg. N) and aerating for 30 minutes; the following results were obtained.

mg. N taken		mg. N estimated
10		9·87
10		9·87
10		9·94
10		10·00
	Mean	9·92

The results for the ammonia in urine have been checked by the recently described colorimetric method of Orr [1924].

Aeration method. mg. ammonia N per 100 cc.	Colorimetric. mg. ammonia N per 100 cc.
55·0	56·3
24·2	24·6
16·18	16·18
39·9	41·0
17·5	17·5

Method for Urea in Urine.

The quantities used are 5 cc. of urine (of known ammonia content), 15 cc. of active urease solution prepared by the method of Folin and Wu, and 5 cc. of sodium pyrophosphate solution. The inner vessel contains 10 cc. $N/2$ H_2SO_4, distilled water and methyl red, as in the case of the ammonia determination. The digestion by the urease is allowed to proceed in a bath at 40° for 20 minutes. Then the sodium carbonate is added and the air current passed for half-an-hour. The rest of the estimation is carried out as described before, $N/10$ alkali being used in the titration of excess sulphuric acid.

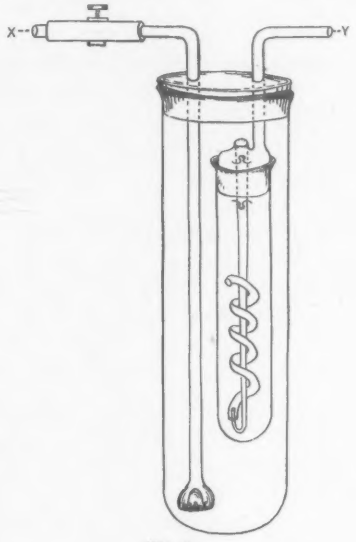

Fig. 1.

These quantities and times have been tested on a standard urea solution of about the same concentration as urine (1 cc. = 10 mg. N).

mg. urea N taken	mg. urea N estimated
100	98·2
100	97·5
100	98·2
100	98·4

Method for Ammonia and Urea in Blood.

A fairly accurate estimation of the urea in blood can be made in a similar way. This is carried out in a smaller form of the apparatus, in which the jacket is 3 cm. in diameter and the inner vessel correspondingly smaller. The volume of blood taken should not be less than 0·6 cc. The proteins are first precipitated by the method of Folin and Wu [1919], and 5 cc. of the clear liquid, which is equivalent to 0·5 cc. blood, are used for the estimation.

I have used 2 cc. $N/100$ H_2SO_4 in the inner vessel to catch the ammonia and titrated the excess acid with $N/200$ NaOH from a burette graduated to 0·02 cc.

For two estimations on a sample of cat's blood I obtained the following values.

Equivalent of blood taken cc.	$N/100$ H_2SO_4 neutralised cc.	mg. urea N per 100 cc. blood
0·5	0·68	19
0·5	0·62	16

The error in this method amounts to not more than ± 4 mg. urea nitrogen per 100 cc. blood.

Although a method for the estimation of urea in 0·2 cc. of blood has been described by Thomas [1924], using $N/400$ acid and alkali, I have been unable to get consistently good results when employing it. This is not difficult to understand, for, if the proteins are precipitated by MacLean's method [1919], the filtrate obtained could not represent more than $\frac{5}{6}$ of the original blood taken, which for a normal blood, containing between 15 and 20 mg. urea nitrogen per 100 cc. blood, corresponds to 0·03 mg. of urea nitrogen. Working with so small a quantity of urea nitrogen, the doubtful quantitative precision of $N/400$ acid and alkali, contained probably in soda glass vessels, to my mind tends to make the method somewhat unreliable.

The extension of Orr's method for the Estimation of Ammonia to the Estimation of Urea in Blood and Urine.

Orr [1924] has recently described a colorimetric method for the estimation of ammonia in urine. I have found the results obtained in this way to agree very closely with those obtained by the aeration method. The estimation is very rapid and, although there are inherent objections to colorimetric methods, it seemed worth while to extend the method to the estimation of urea, since it is usual to have to estimate ammonia and urea in the same sample of urine.

The application of the principles of the colorimetric method to urea estimation was tested, using standard solutions of urea. The details were as follows:

10 cc. of standard urea solution (containing 10 mg. nitrogen per cc.) were pipetted into a 100 cc. graduated flask; 20 cc. of the active urease solution and 10 cc. of pyrophosphate solution were added and the whole made up to

100 cc. The contents of the flask were rapidly transferred to a small conical flask which was very tightly corked and then placed in a bath at 40° for half-an-hour. The flask was then cooled under the tap, the cork raised, and 10 cc. of the solution rapidly pipetted into a 100 cc. flask containing approximately 90 cc. of distilled water; the volume was adjusted to the mark.

The estimation was then carried out with 5 cc. of this solution exactly as described by Orr and using the standard ammonium sulphate (1 cc. = 1 mg. N) for comparison.

This method seems to estimate only 94 % of the total urea and as this figure was very constant with the standard urea solutions, and the values for urea in urine by this method were lower than in the aeration method, it is proposed to make a correction for this in stating results. This low value is no doubt due to inactivation of the urease.

The hydrolysed urea solutions are very easily compared; there is no turbidity due to the enzyme solution and no alteration in the tint of the solution.

If soya-bean meal alone is used the comparison is not so easily made and several times when using the meal I have obtained results much higher than the theoretical value.

Estimation of Urea in Urine Colorimetrically.

10 cc. urine, 20 cc. urease solution, 10 cc. pyrophosphate solution are run into a 100 cc. flask and made up to the mark. This is transferred to a conical flask, corked well and digested in a bath at 40° for half-an-hour. After cooling, 10 cc. of this solution are taken and diluted to 100 cc., as described before. 5 cc. are used for the estimation in the usual way.

Example.

Ammonia N aeration. mg. per 100 cc.	Ammonia N colorimetric. mg. per 100 cc.	Urea N aeration. mg. per 100 cc.	Urea N colorimetric. mg. per 100 cc.	Corrected value for colorimetric. mg. per 100 cc.
55·0	56·3	990	941·5	1000
39·6	41·0	1031	960·0	1021

Estimation of Urea in Blood.

Though the details have been worked out for 0·2 cc. of blood, larger quantities can be used, probably with better results, but for clinical purposes it seems desirable to have a method suitable to this small quantity. If a systematic blood analysis is being made by the Folin-Wu procedure then the estimation is carried out on 2 cc. of the protein-free filtrate.

Method for 0·2 cc. of blood. Place in a small centrifuge tube of about 7 cc. capacity 4·6 cc. of distilled water, run the blood into this from a MacLean 0·2 cc. pipette, washing the pipette out in the usual way. When the blood is laked add 0·6 cc. of 10 % sodium tungstate and then 0·6 cc. of $\frac{2}{3}$ *N* H_2SO_4 (giving a total volume of 6 cc.). Mix and shake till the typical chocolate colour is obtained and heat gently. Centrifuge the tube till the precipitate

is well settled. Then transfer by means of a pipette 5 cc. of the supernatant liquid, which represents $\frac{5}{6}$ of 0·2 cc. of blood, into the outer jacket of the small aeration apparatus described above, add 2 cc. urease extract and 0·5 cc. of pyrophosphate solution. In the inner vessel place 2 cc. of distilled water, two drops of $N/10$ H_2SO_4 and 2 g. of pure phenol. Place the apparatus in a bath at 40°, connect to a pump in the usual way and leave to digest for 10 minutes. Then disconnect, run in 5 cc. saturated Na_2CO_3 solution, replace the apparatus in the bath and aerate for 20 minutes.

Take two 25 cc. graduated tubes; to one which is to be used as the standard add 2 g. of phenol, 4 cc. of distilled water and then 2 cc. of standard ammonium sulphate solution containing 20 mg. nitrogen per 100 cc. Into the other tube empty the contents of the inner tube of the aeration apparatus, wash this tube and spiral with 2 cc. of water and add the washings to the graduated tube. Then to each of the tubes add 5 cc. of sodium hypochlorite, gradually and with shaking, allow to stand for 5 minutes, make each up to the 25 cc. mark and compare at once in a colorimeter with the standard at 30 mm. or 20 mm. as preferred. A stronger standard will sometimes be needed or alternatively the unknown solution can be diluted.

Results. Standard urea solution containing 20 mg. urea N per 100 cc.

Reading for standard ammonium sulphate solution: 20 mg. N per 100 cc. mm.	Reading for the urea solution mm.	Strength of urea solution calculated: mg. per 100 cc.
30	28·7	20·9
30	30·6	19·6
30	31·0	19·0
	Mean	19·8

Results for Blood. A. Using 2 cc. Folin-Wu filtrate ≡ 0·2 cc. blood.

	Standard reading mm.	Blood reading mm.	mg. urea N per 100 cc.
A_1	20	26·6	15
A_2	30	39·6	15
B_1	30	29·6	20

B. Using 0·2 cc. of same blood as used in A_1 and A_2 and precipitating as described.

Standard reading mm.	Blood reading mm.	mg. urea N per 100 cc.
20	29·6	16
30	45·2	15

The colour of these solutions is a clear blue, slightly greener than that obtained with the urine standard, and it is, I think, much more suitable for colorimetric comparison than the yellow of Nesslerised solutions of the same ammonia content. It does not seem possible to use the protein-free filtrate directly in this estimation, for if this solution is digested and then run on to the phenol a green coloration develops on the subsequent addition of the hypochlorite solution.

SUMMARY.

1. Ammonia and urea in urine and blood are estimated by aeration in a simple form of apparatus.

2. Urea in urine is estimated colorimetrically.

3. Urea is estimated in 0·2 cc. blood by combination of the aeration and colorimetric methods.

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XLVI. DETERMINATION OF CARBON MONOXIDE IN BLOOD.

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(*Received January 26th, 1925.*)

Carbon monoxide in blood has been determined in several ways. Except in methods like Haldane's [1895], which is colorimetric, and Hartridge's [1912], which is spectroscopic, a combination of two chemical treatments is necessary; namely, the extraction of the CO from the blood and the subsequent determination of the quantity of the gas.

The determination of CO by means of iodine pentoxide has found a wide application, quantities as small as 0·05 cc. being estimable. The method was first applied to blood by Nicloux [1898], who extracted the CO with the vacuum-pump of Gréhant [1894]. Later on nobody seems to have used iodine pentoxide for this purpose. Plesch [1914] and Gad-Andresen [1916] liberated the CO together with the oxygen in the apparatus of Barcroft or Krogh by means of potassium ferricyanide and determined the CO by combustion.

Van Slyke and Salvesen [1919] liberated the CO together with the oxygen and determined the former gasometrically after absorption of the oxygen.

When, however, the CO content of the blood is less than 10 % of its total capacity these methods are lacking the accuracy necessary in physiological and forensic medical work. The method given by Nicloux may give better results, but he does not give any control experiments on the subject.

By combining Haldane's ferricyanide method with the use of I_2O_5 it is possible to reach a greater accuracy, if one can afford to use at least 10 cc. of blood.

1. *Determination of small quantities of* CO.

CO made in the ordinary way from formic and sulphuric acids was analysed by means of the capillary pipette described by Jordan and Schwarz [1920]. As absorbing fluids were used a solution of pyrogallic acid in strong potassium hydroxide for oxygen and carbon dioxide, and a solution of cuprous chloride in strong ammonia and ammonium chloride for carbon monoxide.

A small quantity of this analysed gas was measured in the Jordan-pipette and sucked into an evacuated flask of one litre capacity. Then outdoor air was introduced and mixed with the CO by shaking, a small quantity of water securing a thorough mixing. The resulting gas mixture was then led over I_2O_5 heated to 150°. This I_2O_5 (5 g.) was contained in a U-tube (*D*) of Jena glass,

being kept in position by small plugs of glass-wool or asbestos. The gas before reaching the I_2O_5 is led first through strong KOH (*A*) and then through strong sulphuric acid (*B*) to be freed from traces of SO_2 and water, which would interfere with the determination of the CO. Tube *C* is a glass spiral to retain traces of sulphuric acid. Ordinary Peligot tubes were used as wash bottles. The iodine liberated is retained in two Peligot tubes (*E* and *F*) containing 0·5 % KI solution, and afterwards titrated with 0·01 *N* thiosulphate from a micro-burette. 1 cc. of 0·1 *N* thiosulphate corresponds to 5·6 cc. of CO.

Before the apparatus is used for determinations the I_2O_5 should be subjected to a current of air devoid of reducing gases at a temperature of about 200° for several hours, until it gives off only traces of iodine at 150°; these traces should be constant in quantity in order that a reliable blank value may be obtained.

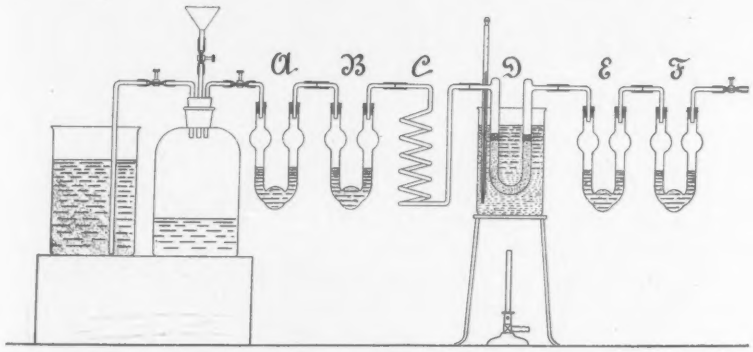

Fig. 1.

Table I indicates amounts of CO taken, as calculated from the values found with the use of the Jordan pipette and those found by titration of the iodine liberated.

Table I.

cc. CO taken	cc. CO found
0·027	0·043
0·060	0·068
0·113	0·122
0·144	0·120
0·149	0·114
0·308	0·319
0·634	0·680
1·21	1·04
1·61	1·31

In the last three determinations the gas was measured by means of a Van Slyke pipette.

2. *The extraction of the* CO *from the blood.*

Van Slyke and Salvesen have pointed out that for extraction of oxygen and CO from blood it is convenient to use ferricyanide together with evacuation. This principle has been applied in the following way. For the extraction a wide-mouthed bottle of 1 litre capacity is used. This bottle is closed by a rubber stopper, through which pass three tubes; two of these are bent at right angles; all three carry rubber tubes, which are closed by screw clamps (see Fig. 1). Attached to the rubber tube of the straight tube is a glass-funnel. The bottle is evacuated by the use of an ordinary suction-pump. Then a measured quantity of blood, *e.g.* 10 cc., is measured into the funnel; by opening the screw clamp below the funnel the blood is sucked into the bottle; after the blood $1\frac{1}{2}$ times its volume of distilled water are introduced in the same way and mixed with the blood by rotating; then through the funnel are added 4 cc. of fresh saturated solution of potassium ferricyanide and mixed with the contents of the flask by vigorous shaking; the flask is now placed in a bath at 40° and shaken at regular intervals during a period of about 30 minutes. There is no need to add ammonia to prevent the escape of CO_2 from the blood; on the contrary, the foaming which accompanies the liberation of CO_2 facilitates the decomposition of the CO-haemoglobin[1].

For the determination of the quantity of CO liberated after the extraction, outdoor air is introduced into the flask until atmospheric pressure is reached and the contents are thoroughly mixed; now one of the rubber tubes of one of the two bent tubes of the bottle is connected with the first Peligot tube (containing KOH) of the apparatus for CO determination, whilst the other is connected to a tube dipping into a jar filled with 2 litres of water. The last Peligot tube of the apparatus (*F*), containing potassium iodide, is connected to a suction pump, which is opened sufficiently to secure a passage of about 1 l. of gas through the apparatus in 30 minutes. When nearly all of the gas has passed, some amyl or capryl alcohol is introduced into the bottle to destroy the foam and the suction of the gas is continued until the bottle is full, when the screw clamp between the apparatus and the pump is closed and the bottle disconnected. The apparatus is now connected with another bottle containing outdoor air of which 1 or 2 l. is led through the apparatus to wash the I_2O_5. The KI solution from the last two Peligot tubes is now titrated, allowing for the iodine which is liberated in a blank determination made by passing 2 or 3 l. of outdoor air through the apparatus.

The usefulness of the technique described was tested in the following way. Fresh horse or ox blood was saturated with CO, and the content of CO determined as described. Then known quantities of this blood were mixed with fresh blood. The CO content of the resulting mixture was again determined and compared with the CO content as calculated. As at first the results were

[1] This was found after most of the control experiments given in Table II had been accomplished; in them dilute ammonia was used.

not satisfactory, it appeared that the fresh blood itself was not always CO-free, probably on account of the air in the stables. Table II indicates the results obtained.

Table II.

	cc. of CO in 10 cc. of blood		cc. of mixed blood		cc. of CO in 10 cc. of blood	
	Fresh	Saturated	Fresh	Saturated	Calculated	Found
1	0·009	1·10	70	3	0·054	0·058
2	0	0·72	90	10	0·072	0·095
3	0·062	1·01	95	5	0·109	0·112
4	0·051	1·46	95	5	0·122	0·121
5	0	1·26	90	10	0·126	0·126
6	0·034	1·49	90	10	0·180	0·200
7	0·069	1·32	90	10	0·194	0·215
8	Not determined	1·21	10	10	0·605	0·614
9	,,	1·29	10	10	0·647	0·685
10	,,	1·32	10	10	0·662	0·602
11	,,	1·29	0	10	1·29	1·23
12	,,	1·29	0	10	1·29	1·29
13	,,	1·32	0	10	1·32	1·32
14	,,	1·49	0	10	1·49	1·45

I am indebted to Prof. W. E. Ringer for much personal help.

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XLVII. SOME REACTIONS OF RESTING BACTERIA IN RELATION TO ANAEROBIC GROWTH.

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(*Received January 12th, 1925.*)

I. Anaerobic growth of facultative anaerobes.

The circumstances in which the facultative anaerobes will grow under strictly anaerobic conditions are far from clear. It is known that the presence of carbohydrates capable of attack by the organism in question favours anaerobic growth; Ritter [1907] has found that, besides carbohydrates, some of the higher alcohols and hydroxy acids are efficient. Nitrates have been found to induce anaerobic growth of facultative anaerobes [Ritter, 1907; Veillon and Mazé, 1910] and Beijerinck [1903] has perceived the possibility that the liberation of energy from nitrate and sulphate oxidations might be utilised by micro-organisms for growth. A mass of data has accumulated on the bacterial reduction of nitrates to nitrites since the discovery of the phenomenon by Schönbein in 1868. Maassen [1901] has shown that 85 cut of 109 types of bacteria tested will bring about the reduction. It is generally accepted to-day that the biological significance of the reduction of nitrate is that by it oxygen is supplied to an organism when free oxygen is no longer available.

The facts, however, are somewhat disconnected and little systematic analysis of the conditions under which anaerobic growth of the facultative anaerobes occurs appears to have been attempted.

II. The action of resting bacteria on nitrates.

Haas and Hill [1923] showed that in milk there was a system which could bring about the reduction of nitrate to nitrite and Dixon and Thurlow [1924] have been able to demonstrate that nitrate will replace methylene blue in the Schardinger reaction and in the xanthine oxidase reaction in milk.

There can be little question that nitrate is capable of acting as a hydrogen acceptor in a manner similar to methylene blue.

What part, then, is played by nitrate when this is present in a bacterial medium? Does it remove hydrogen in the sense that sulphite removes acetaldehyde in Neuberg's fermentations, and thus divert the course of the

normal processes? Or does it play a more significant and positive rôle which would, for instance, be quite distinct from that of a fixative, or of methylene blue considered merely as a hydrogen acceptor?

Resting bacteria have been defined [Quastel and Whetham, 1924] as bacteria which have been grown for two days on tryptic broth, centrifuged, washed with saline and finally very well aerated. They are used under such conditions that multiplication of the cell does not occur. The reactions of the resting bacteria under our experimental conditions are thus due to the non-proliferating organism.

It was found that in the presence of resting bacteria succinic acid donates hydrogen to methylene blue with the production of fumaric acid. It was also found that in the presence of the bacteria fumaric acid oxidises leucomethylene blue, so that finally an equilibrium is established between succinic acid, fumaric acid, methylene blue and leucomethylene blue.

Now it is clear from the oxidising action of fumaric acid upon leucomethylene blue (which does not occur *in vitro*) that the resting bacteria activate the fumaric acid molecule. Activations of methylene blue and leucomethylene blue do not occur, for these are substances which are easily capable of accepting and donating hydrogen respectively *in vitro*, *i.e.* without activation. They form mixtures capable of giving definite oxidation-reduction potentials, measurable at an electrode, and are typical of normal oxidation-reduction systems of which the ferric-ferrous system is an example. Methylene blue and similar substances can therefore be regarded in a sense as already activated *in vitro*. Succinic acid, on the other hand, cannot donate hydrogen *in vitro*. Hence its power of reducing methylene blue *in vivo* must be ascribed to an activation of the acid by the cell. Similarly the oxidising action of fumaric acid *in vivo* must be attributed to an activation of this acid.

As in the case of muscle, there are many substances besides succinic acid which are donators of hydrogen to methylene blue in the presence of resting bacteria (*B. coli* or *B. pyocyaneus*). Among these are lactic acid, β-hydroxybutyric acid, glutamic acid, etc., and a detailed discussion of these will form the subject of a subsequent communication.

The influence of nitrate upon the rate of reduction of methylene blue by various hydrogen donators in presence of resting *B. coli* and resting *B. pyocyaneus* has been investigated. The experimental method was that employed in the work on succinic and fumaric acids [Quastel and Whetham, 1924].

Measurements of the velocity of reduction of methylene blue were made under anaerobic conditions in vacuum tubes at 45° in carefully buffered solutions at a p_H of 7·2.

The observations on methylene blue reductions recorded below are typical of the average of those made. The organism mainly employed for quantitative work was *B. coli comm.*, but *B. pyocyaneus* was sometimes used and found to give similar results.

The first experiments showed that nitrate has a most powerful inhibiting

influence upon the velocity of reduction of methylene blue in presence of the bacteria. (See Table I.)

Table I.

Tubes	(1)	(2)	(3)
cc. of emulsion of resting *B. coli*	1	1	1
cc. phosphate buffer solution p_H 7·2	1	1	1
cc. 1 % Na succinate solution	0·5	0·5	–
cc. 1 % K nitrate solution	–	1	–
cc. methylene blue solution 1/5000	1	1	1
	Reduced completely in 8 mins.	Remained deep blue.	Not completely decolorised in 2 hrs.

It was expected that since nitrate can act as a hydrogen acceptor in a manner similar to methylene blue it would prolong the time of reduction of the dyestuff through competition for the hydrogen. Unless, however, there was a preferential attack of the nitrate, this would be no explanation for the complete inhibition of the reduction of methylene blue. This inhibition resembled that due to fumarates and for further analysis of the phenomenon recourse was had to the technique employed in demonstrating the oxidising action of fumaric acid *in vivo*.

Use is made of an inverted U-tube which can be evacuated. In one limb of the tube is placed a mixture of resting organism, buffer solution, hydrogen donator solution, and methylene blue, and in the other limb a solution of the substance whose oxidising action is to be investigated. The tube is evacuated and, if necessary, filled with nitrogen. The methylene blue is allowed to become completely reduced at 45°, and the solutions in the two tubes are mixed. If there occurs a permanent blueing it is evident that an oxygen donator is present. To show that the oxidation is due to a thermolabile enzyme, the tube containing the reduced methylene blue is heated to any desired temperature before the solutions are mixed. If after heating there is no permanent blueing it is concluded that a thermolabile enzyme controls the oxidation. Blank experiments must of course be made to show that the substance in question does not oxidise leucomethylene blue *in vitro*.

By making use of this technique the following facts were established:

(1) Nitrate oxidises leucomethylene blue in presence of resting *B. coli* or *B. pyocyaneus*, but not *in vitro* (at p_H 7·2 and 45°). In equivalent concentration it is more powerful than fumarate in oxidising the leuco dyestuff.

(2) The oxidation by nitrate is dependent, like that by fumaric acid, on a thermolabile enzyme.

In one limb of a vacuum U-tube was placed a mixture of 1 cc. resting *B. coli*, 1 cc. 1 % sodium succinate solution, 1 cc. phosphate buffer solution, p_H 7·2, and 1 cc. 1/5000 methylene blue solution, and in the other 1 cc. of a 5 % sodium nitrate solution.

The technique was carried out as described above and no oxidation of the leucomethylene blue was found to occur; if the organism had not been heated the oxidation would have been extremely rapid.

(3) Nitrite cannot reduce methylene blue or oxidise leucomethylene blue in presence of resting *B. coli* or *in vitro* (at p_H 7·2 and 45°).

The enzyme dealing with nitrate, like that which deals with succinic acid and fumaric acid, appears to be closely associated with the physical structure of the organism. It is destroyed when the organism becomes coagulated either by heat or by treatment with acid.

These results suggest that when nitrate is added to a nutrient medium containing *B. coli* or *B. pyocyaneus* the nitrate around the cells becomes activated so that it is capable of oxidising substances in the vicinity of the cell (or at the cell surface) which it is incapable of oxidising *in vitro* (or in the body of the medium where the nitrate is outside the sphere of influence of the cell). Now there must be a definite pressure of nitrate molecules on every cell in the medium and since a number of these molecules become activated at the cell surface, each organism will become the centre of a sphere where active oxidations will proceed. Each cell may then be regarded as in a state comparable with that which exists under aerobic conditions.

In this manner the action of nitrate offers a distinct contrast to that of methylene blue, which does not require activation by the organism. Moreover, since the cell surface, like any enzyme, will become "saturated" with regard to the activated nitrate, the oxidation conditions around the cells, above a certain concentration of nitrate, will remain constant. Since nitrite is inert from the point of view of oxidation and reduction (*i.e.* by *B. coli*) and is an easily diffusible ion, its production will not affect the oxidation conditions, and hence the velocity of oxidation of a hydrogen donator at the cell surface. With methylene blue, however, the oxidation potential at the cell surface will depend on the relative proportion of methylene blue to leucomethylene blue and will vary as this proportion alters.

III. The action of resting bacteria on chlorates and perchlorates.

Using the same technique as with nitrate it was found that chlorate is a most powerful (anaerobic) oxygen donator. It will not oxidise leucomethylene blue *in vitro* but a vigorous oxidation occurs in the presence of resting *B. coli* or *B. pyocyaneus*. In the presence of chlorates complete reduction of methylene blue by a hydrogen donator *in vivo* does not appear to take place. Chlorate, moreover, will not effect the reduction of methylene blue by cysteine or glutathione (*i.e. in vitro* reduction); it will do so, however, in the presence of resting organism. As in the case of fumarates and nitrates, the action of chlorates depends on a thermolabile enzyme. (See Table II.)

Perchlorates neither oxidise leucomethylene blue *in vivo* nor *in vitro*. Bromates and iodates both oxidise leucomethylene blue *in vitro*.

IV. The action of resting *B. subtilis* on nitrates and chlorates.

Resting *B. subtilis* was investigated in a similar manner to *B. coli* and *B. pyocyaneus* and it was found that neither nitrates nor chlorates bring about

any perceptible oxidation of leucomethylene blue in presence of the organism. Fumarate oxidises leucomethylene blue in the presence of the organism only very slowly and certain strains do not appear to produce any oxidation. If *B. subtilis* can activate fumarate, nitrate or chlorate at all, the activation is very slight indeed.

Table II.

Tubes	(1)	(2)	(3)
cc. 1 % Na chlorate solution	1	1	1
cc. buffer solution p_H 7·2	1	1	1
cc. methylene blue solution 1/5000	1	1	1
cc. cysteine solution 4 mg./cc.	1	1	1
cc. resting *B. coli*	1	–	–
cc. resting *B. coli* which has been heated at 100° for 20 mins.	–	1	–
	Reduction of methylene blue never complete.	Rapid reduction in 10 mins.	Rapid reduction in 10 mins.

It is well known, of course, that *B. subtilis* will not bring about the reduction of nitrate to nitrite in a fermenting medium, whilst *B. coli* and *B. pyocyaneus* do so very readily.

V. Consideration of some conditions necessary for growth.

It is clear that for an organism to grow energy must be supplied to it for its synthetic operations. This is secured either by the oxidation of the substrate or by its anaerobic breakdown (occurring with the liberation of energy). It has been suggested [Stephenson and Whetham, 1924] that in the anaerobic growth of *B. coli* upon glucose the energy is derived from the anaerobic breakdown of glucose into lactic acid, a change involving the liberation of 22·56 Cal. per gram-mol. of glucose. It was shown, in fact, that even in the presence of air the early period of the growth of *B. coli* upon a glucose medium is anaerobic.

With lactic acid, succinic acid, glycerol, etc., all the likely paths of anaerobic breakdown appear to involve absorption of energy and on none of these compounds, where these formed the sole sources of carbon, and ammonia the sole source of nitrogen, was anaerobic growth of *B. coli* found to occur.

Beijerinck and Folpmers [1916] showed that fumaric acid and malic acid can be broken down by bacteria to pyruvic acid, and one of us [Quastel, 1924] was able to demonstrate that in the growth of *B. pyocyaneus* on ammonium fumarate, the whole of the oxygen absorbed during the first two or three days' growth was accounted for by oxidation of the fumaric acid to oxalacetic (or pyruvic) acid. Within the limits of experimental error no oxygen uptake by the bacteria alone could be demonstrated. In other words the molecular oxygen utilised simply oxidised the fumaric acid to pyruvic acid. The latter molecule entered into the organism's synthetic operations and the necessary energy for synthesis was derived from the fumaric acid oxidation. This point is worthy of emphasis for it clearly demonstrates that given (1) an adequate source of energy, (2) the appropriate molecule to enter into the organism's

synthetic reaction, the presence of molecular oxygen may not be necessary for growth.

There is another point to consider. If the organism is incapable of activating a substrate molecule, *i.e.* if it does not possess a mechanism which will induce the molecule to liberate energy (*e.g.* either by oxidation or by anaerobic breakdown), it is clear that this molecule cannot serve as a basis for a nutrient medium. For instance, if an organism cannot activate fumaric acid so that the latter may become oxidised so as to liberate energy and form pyruvic acid, this organism will not grow upon ammonium fumarate as sole source of carbon and nitrogen.

B. subtilis cannot activate fumaric acid to any appreciable extent (see above) and, as has been previously shown, it does not grow aerobically (or anaerobically) on ammonium fumarate. *B. coli* and *B. pyocyaneus* activate fumaric acid and both grow aerobically upon ammonium fumarate.

The following three conditions at least seem, therefore, to be necessary before growth may occur:

(1) The organism must be able to secure energy either by anaerobic decomposition of the substrate (*e.g.* glucose into lactic acid) or by the oxidation of the substrate.

(2) The organism must be able to activate the substrate so that the latter is capable of reaction, *e.g.* oxidation or reduction, which it does not necessarily undergo *in vitro*.

(3) The products of oxidation (or decomposition) of the substrate, or the substrate itself, must be capable of entering into the synthetic operations of the organism.

It follows that if molecular oxygen is replaced by some molecule which induces around the bacterial cell a state comparable with that which occurs under aerobic conditions, and so long as the three conditions enumerated above are fulfilled, then anaerobic growth should be possible in presence of this molecule.

It has been shown above that we have reason to suppose that nitrate produces about the cell a state comparable with that existing under aerobic conditions. Chlorate and fumarate, which both oxidise leucomethylene blue in presence of resting *B. coli* and *B. pyocyaneus* should therefore have effects similar to those of nitrate. Provided, then, that conditions (1), (2) and (3) enumerated above are complied with, the presence of one of these substances (nitrate, chlorate or fumarate) should enable *B. coli* or *B. pyocyaneus* to grow anaerobically on compounds, such as lactic acid, which will not alone normally support anaerobic growth.

Condition (1) is satisfied by lactate and nitrate, and lactate and fumarate, as the following equations show:

$$KNO_3 + CH_3 \cdot CHOH \cdot COOH = KNO_2 + CH_3 \cdot CO \cdot COOH + H_2O + 30{\cdot}7 \text{ Cals.}$$

$$COOH \cdot CH = CH \cdot COOH + CH_3 \cdot CHOH \cdot COOH = COOH \cdot CH_2 \cdot CH_2 \cdot COOH + CH_3 \cdot CO \cdot COOH + 16 \text{ Cals.}$$

Condition (2) is satisfied as shown by the results described in section II.

Condition (3) is satisfied by the production of pyruvic acid.

Since perchlorate on the other hand is not activated by *B. coli* or by *B. pyocyaneus*, it cannot be expected to produce anaerobic growth or oxidation, and since nitrate is not apparently activated by *B. subtilis* it should not induce the anaerobic growth of this organism.

VI. Anaerobic growth in the presence of nitrates.

Throughout the following experiments the inorganic medium employed was as follows:

0·4 g. $(NH_4)_2HPO_4$.
0·1 g. NaCl.
0·1 g. KH_2PO_4.
0·07 g. $MgSO_27H_2O$.
100 cc. distilled water.

To this was added 1% of a carbon compound.

The solution was brought to a p_H of 7·2 and autoclaved in test-tubes in 10 cc. lots. Each of them was inoculated with 0·1 cc. of a 20-hour broth culture of *B. coli* or *B. pyocyaneus* and incubated aerobically or anaerobically as required. Experience has shown that it is very desirable to inoculate with relatively heavy sowings of the bacteria in order to obtain consistent and regular results.

The results are enumerated below.

A. with *B. coli*.

(1) Growth of *B. coli* occurs under strictly anaerobic conditions in the presence of nitrates, and with pyruvic acid, lactic acid, succinic acid, fumaric acid or glycerol as the sole source of carbon. No growth occurs anaerobically on these substances in the absence of nitrates.

(2) *B. coli* produces from lactic acid aerobically and anaerobically in the presence of nitrates a relatively large quantity of pyruvic acid. The yield is of the order obtained from *B. pyocyaneus* growing upon fumaric acid. *B. coli* when growing aerobically upon lactic acid in the absence of nitrates does not liberate pyruvic acid. This phenomenon will be discussed in more detail later.

(3) Growth of *B. coli* in the presence of nitrates is always accompanied by the production of large quantities of nitrite.

B. with *B. pyocyaneus*.

(1) Extensive growth of *B. pyocyaneus* occurs in the presence of nitrates under strictly anaerobic conditions on citric acid, glucose, lactic acid, or pyruvic acid as sole source of carbon. *B. pyocyaneus* will not grow anaerobically upon these substances (with the exception of glucose) in the absence of nitrates. It will grow anaerobically to some extent on glucose alone without nitrates, but not nearly to the same extent as with nitrates. *B. coli*, on the other hand, grows very well on glucose alone anaerobically and the growth is similar to that obtained when nitrates are present.

B. pyocyaneus both aerobically and anaerobically liberates pyruvic acid from lactic acid in the presence of nitrates. It will produce pyruvic acid from lactic acid aerobically in the absence of nitrates (difference from *B. coli*). The growth of *B. pyocyaneus* upon citric acid and nitrate anaerobically and upon citrate alone aerobically is extremely luxuriant. We have found it very convenient to use a tube of citrate medium (containing no nitrate) inoculated with *B. pyocyaneus* as a test for anaerobiosis. In the presence of a trace of oxygen growth will occur.

Good growth, but not so extensive as with citric acid, occurs anaerobically in the presence of nitrates with succinic acid, fumaric acid, or glycerol as sole source of carbon.

(2) *B. pyocyaneus* breaks down nitrites anaerobically. This is not the case with *B. coli*. (This was originally observed by Maassen [1901].)

C. with *B. subtilis*.

No anaerobic growth of this organism has yet been obtained. This agrees with the observation that *B. subtilis* is incapable of activating nitrate.

It is interesting to note that *B. coli* which will not produce pyruvic acid from lactic acid aerobically in the absence of nitrates, will do so in the presence of nitrates. It has been shown by Mazé [1918] that pyruvic acid is formed from lactic acid by several micro-organisms and we have noted the excellent yields of pyruvic acid produced by *B. pyocyaneus* in a well-aerated medium. In the work on fumaric acid it was shown that *B. pyocyaneus*, but not *B. coli*, will liberate pyruvic acid. It was questioned whether this difference between the two organisms was due to the absence from *B. coli* of a specific enzyme present in *B. pyocyaneus*. Later evidence was brought to show [Quastel and Whetham, 1924] that "the non-appearance of a ketonic acid when *B. coli* is grown on a fumarate medium might be explained as due to differences between the rates of oxidation by growing *B. coli* and growing *B. pyocyaneus* and not to the absence from the former of a specific enzyme capable of producing the ketonic acid." This view is clearly corroborated by the nitrate experiments described above. It also agrees with Mazé's observations on the production of pyruvic acid from lactic acid.

VII. The effect of resting *B. coli* on lactate and nitrate: isolation of pyruvic acid.

20 cc. of a thick suspension of resting *B. coli* were placed in a vacuum tube capable of holding 100 cc. To this were added 10 cc. of a neutral 1 % sodium lactate solution, 10 cc. of a 5 % sodium nitrate solution and 10 cc. of a phosphate buffer solution, p_H 7·2. The tube was evacuated and placed in a thermostat at 45° for three hours. The contents of the tube were then centrifuged and the clear supernatant solution treated with phosphoric acid and warmed. Urea was added to remove all nitrite. After cooling, a freshly-prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid was added, and the *p*-nitrophenylhydrazone of pyruvic acid was separated and identified.

VIII. The effect of resting *B. coli* on lactate and chlorate: production of chlorite and pyruvic acid.

The same procedure was adopted as in the above experiment, using chlorate solution instead of nitrate.

After three hours at 45° the presence of pyruvic acid could be demonstrated by the nitroprusside reaction. It was isolated as the *p*-nitrophenylhydrazone. Some chlorate had been reduced to chlorite. The presence of chlorite could be demonstrated by adding a little potassium iodide to the solution, a little starch solution and finally dilute acetic acid. There was an immediate liberation of iodine. Hypochlorite was not formed since this would have reacted with potassium iodide in neutral or alkaline solutions. The chlorite can be estimated iodometrically in the presence of dilute acetic acid.

The oxidising effect of chlorate upon lactate in presence of resting *B. coli* is more vigorous than that of nitrate for equivalent concentrations. No oxidation of lactate by either nitrate or chlorate occurs *in vitro*.

IX. The course of the production of nitrite and pyruvic acid during the growth of *B. coli* on lactate and nitrate.

B. coli was grown aerobically and anaerobically in an inorganic medium containing 1 % lactic acid as sodium lactate, and 1 % sodium nitrate, and the production of nitrite and pyruvic acid was followed in both conditions.

The tubes were inoculated as usual and incubated aerobically and anaerobically. They were examined in duplicate after varying intervals of time. The nitrite produced was estimated iodometrically. 2 cc. of the medium were placed in a small wash bottle, a little potassium iodide and starch solution were added and nitrogen bubbled through vigorously. Dilute sulphuric acid was added and the iodine liberated was estimated by titration with $N/10$ thiosulphate solution.

The pyruvic acid production was followed by the nitroprusside reaction. To 2 cc. of the medium were added solid ammonium sulphate, a few crystals of sodium nitroprusside and finally 2 cc. of strong ammonia. In the tables below a single plus sign indicates a positive but feeble reaction, whilst a number of plus signs indicates an intense reaction demonstrating the production of a large quantity of pyruvic acid. Table III gives the results of one aerobic series and Table IV gives the results of a parallel aerobic and anaerobic series. The maximum amount of sodium nitrite possible from 1 % sodium nitrate is 0·81 %.

It is generally found that the pyruvic acid liberation by *B. coli* growing in a medium containing 1 % lactate and 1 % nitrate is irregular and feeble until the nitrite produced reaches a concentration equivalent approximately to 0·4 % sodium nitrite. After this concentration is reached the pyruvic acid liberation is increased enormously, whilst the actual amount of growth does not appear to increase.

Table III.

Hours from time of inoculation	Growth	Pyruvic acid produced (examined by nitro-prusside reaction)	p_H	% sodium nitrite produced
15	+ + +	+ +	6·8	0·45
20¾	+ + +	+ + + +	6·8	0·57
31¾	+ + +	+ + + + +	7·1	0·65
41½	+ + +	+ + + + +	7·25	0·76
48	+ + +	+ + + + + (0·3 % by colorimetric comparison)	7·3	0·79
65*	+ + +	+ + + + +	7·4	0·81

* An examination of these tubes showed that practically all the cells present were dead.

Table IV.

Hours from time of inoculation	Growth		Pyruvic acid (indicated by nitroprusside reaction)		% sodium nitrite produced	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
18	+ +	+ +	+	+	0·33	0·34
23	+ + +	+ + +	+	+	0·38	0·38
41½	+ + +	+ + +	+ + + + +	+ + + + +	0·69	0·72
66	+ + +	+ + +	+ + + + +	+ + + + +	0·76	0·77

Moreover this course of events occurs both in aerobic and anaerobic conditions. In other words *B. coli* grows on a mixture of lactate and nitrate in the same way whether the conditions are aerobic or anaerobic.

Pyruvic acid was isolated after approximately 50 hours' growth in the following way. To each tube a little chalk was added and the mixture well shaken and filtered. The filtrate, which was practically free from protein matter, was treated with syrupy phosphoric acid (1 cc. of acid for each tube), excess urea was added, the solution warmed and finally well aerated. When all the nitrite was decomposed, the solution was refiltered, cooled, and treated with a little freshly-prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid. The precipitated hydrazone was filtered through a Buchner funnel, washed with glacial acetic acid and water and recrystallised from dilute alcohol.

It was identified as the *p*-nitrophenylhydrazone of pyruvic acid by its melting point (219°) and mixed melting point.

X. The toxic effect of nitrite upon *B. coli*.

To a series of tubes containing 1 % lactate and 1 % nitrate was added sodium nitrite in varying quantities. These tubes were inoculated with *B. coli* and the growth and amount of nitrite present after 16 hours' incubation were examined in the usual way. The following table gives the results.

Table V.

% nitrite present initially	Growth after 16 hours		% nitrite present after 16 hours		% nitrite produced	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0	+ + +	+ + +	0·45	0·45	0·45	0·45
0·01	+ + +	+ + +	0·30	0·32	0·29	0·31
0·05	+ +	+ +	0·27	0·28	0·22	0·23
0·15	+	+	0·20	0·19	0·05	0·04
0·35	0	0	0·36	0·36	0·01	0·01
0·6	0	0	0·6	0·6	0·0	0·0

The concentration of sodium nitrite toxic to *B. coli* in a 1 % lactate 1 % nitrate medium appears to be about 0·35 %. Although this strength of nitrite causes cessation of growth of *B. coli* it does not prevent resting *B. coli* from activating nitrate and lactate, so that the latter interact and produce nitrite and pyruvic acid. This is illustrated by the following experiment. A series of tubes containing 1 % lactate, 1 % nitrate and 0·4 % nitrite was prepared. To each tube was added 1 cc. of resting *B. coli*. After 20 hours each tube showed an increase in the quantity of nitrite present and pyruvic acid was formed as indicated by the positive nitroprusside reaction. This phenomenon affords an explanation of the fact that much pyruvic acid does not appear during the growth of *B. coli* on a lactate-nitrate medium until the nitrite production has reached about 0·4 %. Below this strength of nitrite the pyruvic acid produced from the lactate is almost entirely used for growth. As soon, however, as the nitrite reaches its toxic concentration and prevents further bacterial synthesis, the pyruvic acid, which is still being produced from the lactic acid by the action of nitrate and the resting organism, is diverted from synthesis and is liberated into the medium.

XI. The breakdown of nitrite by *B. pyocyaneus*.

The breakdown of nitrite by *B. pyocyaneus* is a well-known phenomenon. The following experiment shows the ease with which it is effected.

A medium containing 1 % lactate and 0·1 % nitrite and another containing 1 % lactate and 0·05 % nitrite were sterilised and sown with 0·1 cc. of a 20-hour broth culture of *B. pyocyaneus* and incubated aerobically. The following table gives the results.

Table VI.

Hours after time of inoculation	0·05 % nitrite (as K salt) medium	0·1 % nitrite (as K salt) medium
21½	All nitrite utilised	0·03 % nitrite still present
41½	—	All nitrite utilised

XII. Aerobic and anaerobic growth of *B. coli* in the presence of chlorates.

So far it has been found impossible to substitute chlorates for nitrates in anaerobic growth, although the organism has been shown to be capable of activating chlorate. This failure of chlorate to induce anaerobic growth is probably due to the production of chlorite (p. 312), which is toxic to *B. coli* at a concentration of 1 in 20,000.

The following series of experiments (see Table VII) shows the toxic effect of chlorate on *B. coli* growing on lactate and nitrate.

Tubes containing 1 % lactate, 1 % nitrate and varying quantities of potassium chlorate were inoculated in the usual way and incubated at 37° both aerobically and anaerobically.

Table VII.

% $KClO_3$	0·5	0·1	0·025	0·005	0·001	0
Aerobic.						
Growth after 16 hours from time of inoculation	0	0	?+	+++	+++	+++
Growth after 44 hours from time of inoculation	+	+	+	+++	+++	+++
Pyruvic acid production (indicated by nitroprusside reaction)	++	++	++++	++++	++++	+++
Anaerobic.						
Growth after 44 hours from time of inoculation	0	0	+	+++	+++	+++
Pyruvic acid production (indicated by nitroprusside reaction)	0	0	0	++++	++++	+++

The concentration of potassium chlorate, which is completely toxic to *B. coli* anaerobically under these conditions, is about 0·03 % or 1 in 3000. Aerobically there is a definitely toxic effect of chlorate at about this concentration, but there is slight growth at concentrations of chlorate much higher than 0·03 %.

XIII. The toxic effect of chlorites.

Potassium chlorite was prepared according to the method of Bruni and Levi [1915]. The chlorite obtained was estimated iodometrically (in the presence of dilute acetic acid) and the solution made up with distilled water to 1 %. It was brought to p_H 7·2. The solution was not autoclaved since considerable loss of chlorite is occasioned by so doing—moreover, it is unnecessary since chlorite is a most powerful antiseptic.

Tubes containing 1 % lactate 1 % nitrate medium were prepared and sterilised; to these were added varying quantities of chlorite and the tubes were inoculated with *B. coli*. They were incubated aerobically and anaerobically. Table VIII gives the results.

Table VIII.

% $KClO_2$	0·07	0·05	0·03	0·01	0·005	0
Aerobic.						
Growth after 24 hours	0	0	0	0	(+)	+++
„ „ 48 „	0	0	0	0	(+)	+++
Anaerobic.						
Growth after 48 hours	0	0	0	0	(+)	+++

The fact that resting *B. coli* has been shown to produce chlorite from chlorate and the fact that chlorite is toxic at extremely low concentrations explains why chlorate is ineffective as a substitute for nitrate in anaerobic growth.

XIV. Aerobic and anaerobic growth of *B. coli* in the presence of perchlorates and of maleic acid.

Since perchlorate and maleate [Quastel and Whetham, 1924] do not appear to be activated by resting *B. coli* it was expected (1) that perchlorates and maleates would not act as substitutes for nitrates in anaerobic growth; (2) that perchlorates and maleates would not be toxic to *B. coli* growing upon lactate and nitrate.

Experiment shows that neither perchlorates nor maleates will induce anaerobic growth and that they have no perceptible toxic action on *B. coli* growing upon lactate and nitrate, under aerobic or anaerobic conditions.

XV. Aerobic and anaerobic growth in the presence of fumarate.

If the general conceptions which have been outlined above are correct it should follow that for anaerobic growth nitrates may be replaced by some organic hydrogen acceptor or oxygen donator, so long as this substance is activated by the organism and there is the necessary liberation of energy, etc.

Fumaric acid was chosen as a suitable hydrogen acceptor and experiment showed that *B. coli* will grow anaerobically (in the absence of nitrates) on a mixture of lactate and fumarate where these substances form the sole sources of carbon and ammonia the sole source of nitrogen. On lactate alone and on fumarate alone no anaerobic growth of *B. coli* occurs. For experiment the following solutions were made up:

A. 1 % lactic acid as Na salt, 1 % fumaric acid as Na salt, and the usual inorganic medium.

B. The same with 2 % fumaric acid.

These were autoclaved, inoculated as usual, and incubated aerobically and anaerobically. The results are indicated in Table IX.

Table IX.

	1 % lactate, 1 % fumarate medium	1 % lactate, 2 % fumarate medium
Aerobic.		
Growth after 48 hours	—	Very good throughout medium
p_H after 48 hours	—	7·0
Growth after 72 hours	Very good throughout medium	Very good throughout medium
p_H after 72 hours	7·6	7·0
Anaerobic.		
Growth after 48 hours	—	Very good throughout medium
p_H after 48 hours	—	6·8
Growth after 72 hours	Very good throughout medium	Very good throughout medium
p_H after 72 hours	6·8	6·8

When *B. coli* grows aerobically on fumarate or anaerobically on a mixture of fumarate and nitrate the solution becomes alkaline ($p_H > 7·4$) owing to the decarboxylation of the oxidised fumaric acid. Since lactic acid is a monobasic acid the growth of *B. coli* upon this does not produce immediate alkalinity

but a slight acidity owing to the ammonia which is utilised being more than equivalent to the alkali set free by the utilisation of the lactic acid. Hence, if in a mixture of lactic and fumaric acids there is produced immediate alkalinity after growth of *B. coli*, this would show that fumaric acid is being utilised; if an acidity, this would be evidence for the utilisation of lactic and the non-utilisation of fumaric acid. In all the anaerobic growths of *B. coli* on mixtures of lactic and fumaric acids there is produced slight acidity indicating the utilisation of lactic acid and that the fumaric acid is acting solely as hydrogen acceptor. This was further confirmed by the isolation of succinic acid from these media.

For experiment, twenty tubes, each containing 10 cc. of a 1 % lactate, 1·25 % fumarate medium, were inoculated with *B. coli* and incubated anaerobically for 12 days. All tubes showed good growth; the p_H of the solution was 6·9. The contents of the tubes were mixed and 20 cc. of syrupy phosphoric acid were added. The acid solution was extracted with ether and the white residue from the ether extract dissolved in a little water, silver nitrate solution added, the mixture filtered, and the clear filtrate just neutralised with sodium hydroxide. There was a copious precipitate, which, on decomposition by sulphuretted hydrogen, yielded a solution which did not reduce permanganate and gave a crystalline residue of succinic acid, identified by the melting point (185°) and by a mixed melting point.

It is specially noteworthy that fumaric acid can play a dual rôle:

(1) It will act as an oxygen acceptor and become a nutrient carbon source, *e.g.* in the anaerobic growth of *B. coli* on fumarate and nitrate.

(2) It will act as a hydrogen acceptor and so allow lactic acid (for instance) to be utilised, *e.g.* in the anaerobic growth of *B. coli* on a mixture of fumarate and lactate.

We wish to express our appreciation of the continued interest of Sir F. G. Hopkins in this work. Two of us (M. S. and M. D. W.) have been enabled to pursue these investigations through grants received from the Medical Research Council.

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XLVIII. THE INFLUENCE OF BLOOD SERUM AND OF SUGARS ON HAEMOLYSIS.

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(*Received March 9th, 1925.*)

Of late considerable interest has been shown in the phenomenon of inhibition of haemolysis, a matter of no little importance. The experiments of Ransom [1901] have been widely quoted, and passed from book to book, as though worthy of complete reliance. His conclusion that the inhibition of saponin haemolysis is due to the cholesterol content of the serum is negatived, however, by the fact that the quantity of cholesterol in serum is not sufficient to bring about the effects. Stocks [1919] more recently investigated the question, and concurs with Ransom, but his results are directly at variance with those of Ponder [1923], who has shown that the protein content of the serum is the chief factor in the production of inhibition. Stocks also believes that the inhibition is the result of a simple chemical reaction, but it can easily be shown that the inhibitory process is a much more complicated one than he would make out. It has therefore been thought well to confirm the findings of Ponder, who has investigated only the action of serum and of simple proteins, and to find out whether other inhibitory substances conform to the particular relation described by him.

Method.

The method adopted is the neat and simple one devised by Ponder, whose papers may be consulted [1921, 1922].

The materials used require some note. The haemolysin is pure white saponin dissolved in 0·85 % NaCl. The cell suspension is one of human cells, prepared by drawing off 1 cc. of blood into citrated saline, washing the cells three times, and then suspending them in 20 cc. of 0·85 % NaCl. A fresh suspension is made up every day. The sera employed were obtained from the clotted blood of the animal concerned, and were used fresh. The sugar solutions were made from pure sugars, dissolved in distilled water, and were isotonic with the cells.

Technique.

The technique of Ponder was closely followed, the result being the production of two curves for each sugar, one showing the relation between the time for the production of complete haemolysis and the dilution of haemolytic

agent in the absence of any inhibitory substance, and the other the relation in the presence of the sugar or serum. The logarithm of the difference between the δ coordinates of the two curves at any time t, is then plotted against the logarithm of the δ coordinate of the lower of the two curves at time t, the dilutions being first converted into concentrations. The result of such a plotting is a straight line as shown in Fig. 2. A typical experiment is set out in the following table; δ_1 is the dilution of lysin employed, c_1 its corresponding concentration, T the time for haemolysis, and δ_2 the dilution of lysin which is

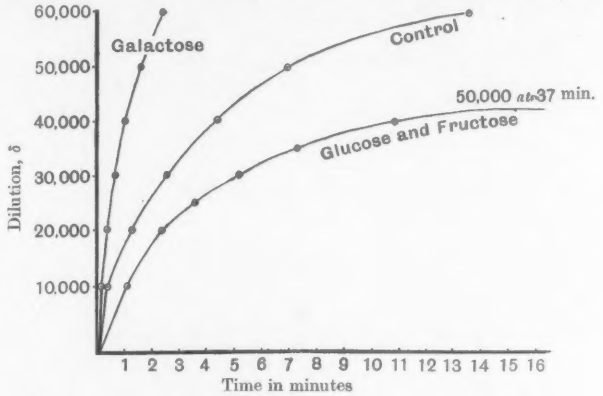

Fig. 1

considered as acting on the cells and not inhibited; c_2 is its corresponding concentration. From these figures the curves shown in Figs. 1 and 2 are derived. In this case glucose is the inhibitory substance.

δ_1 1 in	c_1 mg.	T min.	δ_2 1 in	c_2 mg.	x mg.	log c_2	log x
10,000	0·2	1·0	18,000	0·11	0·09	$\bar{1}$·04	$\bar{2}$·95
20,000	0·1	2·4	30,000	0·06	0·03	$\bar{2}$·82	$\bar{2}$·52
25,000	0·08	3·5	36,000	0·055	0·027	$\bar{2}$·74	$\bar{2}$·43
30,000	0·06	5·1	43,000	0·046	0·02	$\bar{2}$·66	$\bar{2}$·30
35,000	0·057	7·2	50,000	0·04	0·017	$\bar{2}$·60	$\bar{2}$·23
40,000	0·05	10·7	57,000	0·035	0·015	$\bar{2}$·54	$\bar{2}$·17

Results.

(1) *Serum.* For these experiments rabbit serum was used in a dilution such that 0·01 cc. was present in each tube with the saponin. The following results were obtained:

Animal	Value of n	Value of A
1	0·93	1·2
2	0·93	1·09
3	1·0	1·0
4	1·3	0·4
5	0·9	1·12

Ponder gives only one case of the rabbit, in which the constants are $n = 1{\cdot}125$, $A = 1{\cdot}02$; it will be seen that all the above results give considerably lower values of n than the figures for the sera of the cat and of man, so that, for

identical dilution, rabbit serum appears to be less inhibitory. The errors of these values are exceedingly slight, and no difficulty was found in any case in reducing them to the linear form.

(2) *Glucose and Fructose.* Very pure samples were used, and the solutions made up fresh every time they were required. No effect on the cells was observed as a result of the sugars *per se.* It was found that glucose and fructose acted identically when in the same concentration, the curves being so nearly coincident that the one given by fructose has been omitted from

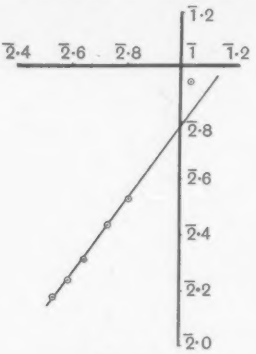

Fig. 2

Note. The uppermost point lies somewhat off the line, but this divergence is really insignificant, and is occasioned by its greater liability to experimental error, and to the sensitiveness of logarithms in this region.

the graph in Fig. 1. It will be seen that the effect of these sugars on saponin haemolysis is substantially the same as that of serum, the linear form of graph being obtained by plotting log x against log c_2. The values of the constants are $n = 0{\cdot}77$, $A = 1{\cdot}0$. This value of n is much less than is usually found in serum experiments.

(3) *Galactose.* A very different effect was here observed. The action is to accelerate in a marked manner the haemolytic rate, as can be seen from the uppermost curve in Fig. 1, although the sugar has no effect on the cells *per se.* So far no method of analysing the acceleration curve has been devised.

Careful experiments were conducted to discover whether the acceleration effect was in any way comparable to the accelerations met with in systems of bile salts and serum, or bile salts and histamine. In such systems the histamine or serum acts as an inhibitor if added to the cell suspension before the glycocholate, and as an accelerator if added after the cells are mixed with the lysin [Ponder, 1922]. This was not found to be so in the galactose-saponin system, nor indeed, was it expected to occur, since no such inhibition-acceleration effects have been observed with saponin, which shows either a clear inhibition as with serum, or a clear acceleration as with aspartic acid and allied substances.

(4) *Starch.* Further experiments were carried out with a highly purified form of rice starch, in many dilutions, but no effect on the saponin haemolysis was observed.

It is not proposed to discuss these results, which are in part confirmatory ones, nor can the reason for the acceleration produced by galactose be dealt with. It is however interesting to note that two such closely allied sugars as glucose and galactose should have such diametrically opposed effects on the action of saponin.

SUMMARY.

1. Certain results are recorded confirming Ponder's results on the inhibition of saponin haemolysis by serum.

2. Glucose and fructose inhibit saponin haemolysis in a manner much like that of serum.

3. Galactose accelerates the action of saponin, and starch has no effect thereon.

This research was carried out during the tenure of a Carnegie Scholarship.

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XLIX. THE RELATION BETWEEN SULPHUR AND NITROGEN METABOLISM.

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(*Received January 12th, 1925.*)

The purpose of the present paper was an attempt to elucidate the following controversial points in protein metabolism.

1. Is the breakdown of ingested protein immediate or is it spread over several days?

2. Is there any difference in the rates at which nitrogen and sulphur are excreted?

3. If ingested protein can be retained is it capable of covering the breakdown of body protein in the first few days of starvation?

4. What is the nature of the stored material?

Is ingested protein dealt with immediately? This question was repeatedly investigated by the older workers, but it was not until the introduction of the superimposition method of Falta that clear proof was obtained that a real delay in excretion may take place. Falta [1906] gave a subject a constant diet until nitrogen equilibrium was established and then on one day superimposed on this diet a known amount of the protein to be investigated. The course of its excretion was then followed for several days. The amount of the protein excreted in each particular day was found by subtracting the total nitrogen output on the basal diet from the nitrogen output on the day concerned. He found that the time of excretion varied with different proteins. Hämäläinen and Helme [1907], using this method, found the same delay in the metabolism. The general conclusion is that there is a retention in some form of the nitrogen of the food.

Is there any difference in the rate at which the nitrogen and sulphur of the ingested protein is excreted? Feder [1881], by following the outputs of nitrogen and sulphur in 2-hourly samples of the urine of a dog after meat ingestion, found an early excretion of sulphur and a delay in the nitrogen output. He also emphasised the importance of 2-hourly samples as opposed to the 24-hourly collection. Falta [1906] found, taking the 24-hour urine, an early excretion of sulphur after the ingestion of egg white and caseinogen. Hämäläinen and Helme [1907] obtained the same result with egg white, veal and proton (caseinogen). Wolf and Osterberg [1911] maintained that when egg white, either raw or coagulated, was superimposed sulphur was more slowly excreted

than nitrogen in the early hours. Lewis [1916] found a lag in the sulphur output as compared with the nitrogen on feeding meat to dogs after starvation. Cathcart and Green [1913], on the other hand, observed a nitrogen lag after the ingestion of gelatin and egg white. The evidence then as regards the rate of excretion of sulphur and nitrogen is on the whole rather contradictory. In regard to the nature of the material stored there has been a certain amount of inconclusive work. Falta thought the variation in the rate of excretion of the nitrogen and sulphur after the ingestion of different proteins was due to the differences in their resistance to breakdown. Ehström [1906], on the other hand, considered that the material was stored in some easily metabolisable form. Obviously, however, if there are differences in the rate at which nitrogen and sulphur are excreted then the stored material must differ in composition from the protein ingested. In the present experiments an attempt has been made to elucidate the problem by starving the subject after the superimposition of some definite protein and following the nitrogen and sulphur outputs.

The experiments described in this paper fall into four groups. These were all carried out on myself, a healthy subject weighing 60 kg. The first series was designed to find the effect of two different proteins on the nitrogen output during the first few days of starvation. In order to eliminate the effect of previous food a nitrogen-free diet was taken until the nitrogen excretion for 24 hours was below 3 g., which is about the lowest that can be reached. Then the protein to be investigated was superimposed for one day. Two days of starvation followed and on the third day the basal diet was resumed until the nitrogen output had fallen to the original value. In order to have a basis of comparison a starvation experiment was carried out without the superimposition of protein: this constituted Exp. 1 (Table I). In Exps. 2 and 3 gelatin and egg white respectively were the proteins superimposed. These two proteins were chosen because of the marked contrast in their sulphur content or S : N ratio. The nitrogen-free diet consisted of olive oil, 100 g., tapioca, 300 g., sugar, 100 g.

The second group consisted of two experiments in which gelatin and egg white were superimposed on a basal diet containing 6 g. nitrogen. The third group was the same except that the basal diet contained about 11 g. nitrogen. The fourth was the same as the first except that no starvation followed the taking of the proteins. In all these experiments the 24-hour output of urine was collected under toluene and analysed on the following day. Total nitrogen was determined by the Kjeldahl method, urea and ammonia by the urease method, phosphorus by the uranium acetate method and uric acid by the Hopkins-Folin method.

Referring to Table I it will be noted that the urinary constituents fell more or less evenly for the first few days. Taking the S : N ratios, however, a distinct rise is seen from 1 : 15·8 on the first day to 1 : 12·3 on the day before starvation. This means that when the body is on a nitrogen-free diet and

Table I.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
9 Jan.	Basal	4·788	0·3640	450	1·7892	0·3022	1 : 15·8	1 : 2·6
10 „	„	4·382	0·4134	327	1·8316	0·2748	1 : 15·9	1 : 2·3
11 „	„	3·052	0·3080	212	1·4200	0·2528	1 : 12·0	1 : 2·1
12 „	„	2·996	0·3024	210	1·1644	0·2418	1 : 12·3	1 : 2·5
13 „	Starving (Exp. 1)	2·492	0·1848	205	0·7524	0·2143	1 : 11·6	1 : 3·3
14 „	„ („)	4·872	0·3360	290	1·3488	0·4286	1 : 11·3	1 : 3·6
15 „	Basal	6·944	0·3808	337	1·2498	0·4012	1 : 17·3	1 : 5·5
16 „	„	4·480	0·3966	225	1·4340	0·2638	1 : 16·9	1 : 3·1
17 „	„	2·996	0·3920	257	1·1369	0·2857	1 : 10·4	1 : 2·6
18 „	Gel. supsd (Exp. 2)	5·901	0·5460	307	0·8520	0·2473	1 : 23·8	1 : 6·9
19 „	Starving	3·984	0·2352	327	1·0648	0·2693	1 : 11·0	1 : 3·7
20 „	„	6·804	0·3024	302	1·4624	0·4836	1 : 14·0	1 : 4·6
21 „	Basal	8·284	0·5656	437	1·0224	0·5660	1 : 14·5	1 : 8·1
22 „	„	4·228	0·3024	262	0·9088	0·2528	1 : 16·7	1 : 4·6
23 „	„	3·332	0·2912	285	0·9228	0·2253	1 : 14·7	1 : 3·6
24 „	Alb. supsd (Exp. 3)	3·920	0·4088	312	0·7952	0·4876	1 : 8·0	1 : 4·9
25 „	Starving	5·628	0·2800	365	0·7808	0·5660	1 : 9·9	1 : 7·3
26 „	„	7·112	0·3360	292	1·4484	0·4506	1 : 15·7	1 : 4·9
27 „	Basal	7·532	0·4368	415	1·1070	0·5056	1 : 14·8	1 : 6·8
28 „	„	3·900	0·2912	299	0·8662	0·2693	1 : 16·7	1 : 4·5
29 „	„	2·996	0·2856	270	0·9230	0·2583	1 : 11·5	1 : 3·2

drawing on its tissue protein there is either a breakdown of some sulphur-rich protein or the retention of the nitrogen of some protein of average sulphur content, *e.g.* muscle. If, however, there were a breakdown of, say, muscle tissue along with a nitrogen retention, this nitrogen should ultimately be excreted and bring down the S : N ratio. This is exactly what happened, as, on the return to the basal diet, the nitrogen rose almost 50 %, while the sulphur output fell a little. Corresponding to this the S : N ratio fell to 1 : 17, but by Jan. 17th rose again to 1 : 10·4. If the total nitrogen and sulphur output of the two starvation and two following days be taken, the S : N ratio of the sum is 1 : 14·3, which corresponds to the average value found for muscle. Apparently then there is either an alternating breakdown of sulphur-rich and sulphur-poor protein or else a breakdown of some protein such as muscle with a nitrogen retention. The sudden elimination of the retained nitrogen on the first day after starvation is probably due to the stimulating action of the food.

Had food been taken constantly the excretion of this retained nitrogen would have increased until the S : N ratio was 1 : 14·3. At this point the body would have a certain quantity of temporarily retained nitrogen in it but its excretion would equal the rate of formation and hence a S : N ratio of 1 : 14·3 would result if muscle were the source. The same rise in the S : N ratio was seen in Cathcart's subject [1907]. The S : N ratio rose from 1 : 14·7 on the last day of starvation to 1 : 9·9 on the third day of a nitrogen-free diet.

Both these experiments, however, show that the influence of carbohydrate on the sulphur moiety is more marked than on that of the nitrogen. This is to be expected, for, if the sulphur of the protein molecule is the first to be broken down and eliminated, the sparing effect of carbohydrate on protein breakdown should be directed primarily at the sulphur-containing group.

From the data of Exp. 1 it is possible to calculate the nitrogen and sulphur loss due to starvation alone by subtracting the basal value (the nitrogen and sulphur output on the day before starvation) from the total nitrogen for each day on which the nitrogen output exceeds the basal value. In this case the two starvation and two subsequent days are involved. The first starvation day gives a positive balance however.

Excess nitrogen output above basal value = 6·804 g.
„ sulphur „ „ „ „ = 0·3646 g.
S : N = 1 : 18·6.

This low ratio does not necessarily mean that sulphur-poor protein has been used for starvation metabolism. The explanation is that on the one hand the S : N ratio of the basal value is high, which shows up the early excretion of sulphur and the retention of the nitrogen. The S : N ratio of 1 : 18·6 on the other hand shows up the elimination of the retained nitrogen. As was stated above, the S : N ratio of those four days is 1 : 14·3. The conclusions drawn from this experiment are:

1. That the sulphur fraction of the protein molecule is broken down and eliminated in advance of the nitrogen. This applies to the breakdown of tissue protein when on a carbohydrate-fat diet.

2. The sparing action of carbohydrates on the breakdown of body protein is exerted first on the sulphur-containing moiety. Carbohydrate has no effect on the temporarily retained nitrogen and this cannot be used for synthesis as there is no adequate supply of sulphur present to make up the full complement necessary for the formation of protein.

The same procedure was carried out in Exp. 2, with the exception that 70 g. gelatin containing 11·8 g. nitrogen and 0·3367 g. sulphur with a S : N ratio of 1 : 35 were superimposed on the day before starvation. It will be noted that the same basal value as in Exp. 1 had been reached on the day before gelatin was taken. Gelatin was chosen as a type of sulphur-poor protein, as will be seen from the S : N ratio. On the day of superimposition the S : N ratio fell to 1 : 23 instead of rising. There is no advanced output of sulphur even, although the S : N ratio for the day (1 : 23) is higher than that of the gelatin taken, because the total sulphur output on the day of superimposition is actually lower than that of the previous day. The sulphur has been completely stored, while some of the nitrogen has been eliminated. On the first starvation day the S : N ratio showed a sharp rise to 1 : 11. The probable explanation of this change is that the first day of starvation produces a very definite reduction in the material metabolised. The nitrogen drops considerably, while the sulphur shows a slight rise. This slight rise is probably due to the metabolism of protein stored the previous day—possibly in an unstable form. Evidence of such an instability will be seen in later experiments. It is not considered, however, that the low S : N ratio on the day of superimposition and the high ratio the following day are to be attributed to a sulphur lag.

The explanation suggested is that in the storage of protein the sulphur fraction is stored first and the nitrogen moiety later. In other words it is the theory of the nitrogen lag applied to the anabolism of protein as well as to its catabolism. One can imagine the movements of the nitrogen fraction always following those of the sulphur. In protein catabolism the sulphur is eliminated first and then the nitrogen follows, whereas in protein anabolism the sulphur is built up first, then the nitrogen. When the influence of carbohydrates on the breakdown of body protein is considered, the effect is seen first on the sulphur output and later on the nitrogen output (Exp. 1). It will be noted that on the return to the basal diet the nitrogen and sulphur outputs both rose proportionately, as there was little change in the S:N ratio. The S:N ratio fell, however, the next day to 1:16·7, so that evidently there was some retained labile nitrogen in the body, though the S : N ratio never returned to its original high value. It is probable that the intake of sulphur-poor protein in the form of gelatin has saturated the tissues to their maximum capacity for holding sulphur-free nitrogen bodies; hence the failure to reach the previous high value of the S : N ratio. If these figures are treated as in Exp. 1 we find:

Excess nitrogen above basal value	= 14·557 g.
„ „ „ „ „ in Exp. 1 (pure starvation)	= 6·804 „
„ „ „ that in Exp. 1 = 14·557 − 6·804	= 7·753 „

Hence by taking 11·8 g. nitrogen in the form of gelatin before starving, 7·753 g. nitrogen were excreted above the output due to starvation alone, and hence 4·047 g. nitrogen were stored or utilised to cover the breakdown of body protein in starvation. In the case of the sulphur output the same calculation is made.

Excess sulphur above basal value	= 0·3704 g.
„ „ „ „ for Exp. 1	= 0·3646 „
„ „ „ that in Exp. 1	= 0·0580 „

As 0·3363 g. were ingested it follows that 0·2783 g. were stored, or covered the sulphur output in starvation. The S : N ratio of the stored material is 1 : 14·5, while that of the gelatin was 1 : 35. If one be permitted to draw a deduction from the nitrogen and sulphur content there would seem to have been a selection among the various amino acids in gelatin in order to retain a substance corresponding to muscle protein.

In the case of phosphorus the total output in Exp. 2 is less by 0·0288 g. P_2O_5 than in Exp. 1. Taking the total nitrogen output in Exp. 1 from the day before starvation to the second post day we find it to amount to 21·77 g. nitrogen. In Exp. 2 it amounts to 29·8 g. nitrogen, but as 11·8 g. nitrogen were ingested the real loss to the body is 29·8 − 11·8 = 18 g. This is 3·77 g. nitrogen less than in Exp. 1; the stored phosphorus is 0·0288 g. P_2O_5 and the P_2O_5 : N ratio of this is 1 : 130. From these figures it can be seen that 58 % of the excess nitrogen output due to starvation can be spared by gelatin.

This is a higher value than those found by the older workers, and is possibly due to the different method of calculation employed. The method of calculation used in the present experiments narrows the question to finding the effect of gelatin on the excess output of nitrogen above the basal value due to starvation alone.

Experiments on the capacity of gelatin to cover the minimum wear and tear output on a nitrogen-free diet were carried out by Robison [1922]. He found that gelatin had very little sparing effect even although a large excess was taken. It would seem to follow that protein of a more specific nature is required to cover the endogenous wear and tear than to cover the excess output above this occasioned by starvation.

Exp. 3 consisted in superimposing egg white under the same conditions. Unfortunately in this experiment the basal value was a little higher on the day before superimposition than on the corresponding day of the previous experiment. 495 g. egg white, containing 8·87 g. nitrogen and 0·960 g. sulphur, with a S : N ratio of 1 : 9·2, were distributed evenly over the three meals on the day of superimposition. The egg white was obtained directly from fresh eggs and taken in the soft-boiled state. It will be noted that this is a sulphur-rich protein. On the day of superimposition the nitrogen output rose 0·59 g., while the sulphur output was nearly double that of the previous day. The S : N ratio accordingly rose to 1 : 8. On the first day of starvation the sulphur and nitrogen outputs both rose, which is in marked contrast to Exps. 1 and 2, where a fall was the rule. A possible explanation of this variation will be found in some of the later experiments. It is probably associated with a peculiar instability of egg white. The S : N ratio on this day is still high. On the second day of starvation the S : N ratio fell to 1 : 15·7, the sulphur output falling a little, while the nitrogen output rose. On the first post day, when the basal diet was resumed, there was the same rise in the nitrogen output though much less than in the previous experiments. The sulphur output also rose a little, but the S : N ratio fell to 16·7, apparently an excess output of nitrogen in relation to sulphur. On the last day the ratio rose to 11·5. In all three experiments a certain amount of temporarily retained nitrogen seems to be eliminated after starvation, but in Exps. 2 and 3 this occurs on the second post day, as deduced from the low S : N ratio, whilst in Exp. 1 it occurs mostly on the first post day. Owing to the fact that the original basal value was not reached in this experiment, there are two figures on which to base the calculations, either the actual basal value obtained for this experiment or the original one of 2·9 g. nitrogen. Taking the former we get:

Excess nitrogen above basal (3·3 g.)	= 11·432 g.
,, ,, ,, ,, in Exp. 1	= 6·804 ,,
,, ,, ,, that in Exp. 1 = 11·432 − 6·804 =	4·628 ,,

Hence 8·87 g. nitrogen in the form of egg white caused an excess output above that found in pure starvation of 4·628 g. nitrogen. As 8·87 g. nitrogen

were ingested it follows that 4·142 g. nitrogen were stored, or covered the breakdown of body protein during starvation.

Excess sulphur above basal	= 1·1792 g.
„ „ „ „ in Exp. 1	= 0·3646 „
„ „ „ output in Exp. 1	= 0·8146 „

As 0·960 g. sulphur was ingested, 0·1454 g. was retained or covered the breakdown of body sulphur. The S : N ratio of the retained material is 1 : 31·7 —a low value. The storage of sulphur is only 0·1454 g. in this experiment, while in Exp. 2 with gelatin it was almost twice as much. It is rather an anomaly that the sulphur retention should have been so low with the sulphur-rich protein as compared with the sulphur-poor gelatin. It is unlikely that some other limiting factor is associated with egg white, such as a minimal percentage of some essential amino acid, especially as it is a much more perfect protein than gelatin. There is the possibility, however, that egg white brought about the temporary retention of some unstable sulphur-rich protein which was quickly broken down. It has recently been shown to contain only 15 % of its sulphur in the form of cystine, so that the effective content of egg white in this amino acid might be low [Harris, 1923]. This presupposes that cystine is the only form of sulphur which the body can utilise. It is questionable however if the sulphur in gelatin is in the form of cystine [Dakin, 1920], though it was, nevertheless, well utilised. The percentage retention of the nitrogen of the white was nearly 50 %, whilst in the case of gelatin it was only 31 %. There is, however, an inconsistency in the above calculations. The excess for this experiment was calculated with 3·3 g. nitrogen as a basal value. The excess output was then compared with the control Exp. 1 in order to see the effect of the egg white on starvation. The excess output on pure starvation (Exp. 1) was calculated on a basal figure of 2·9 g. nitrogen. Hence, to make the comparison more valid, the excess output for pure starvation should be recalculated with 3·3 g. nitrogen as a basal figure. This result should then be subtracted from the excess output found in Exp. 3 with 3·3 g. nitrogen as a basal figure. This recalculation gave an excess output for pure starvation of 5·66 g. nitrogen. The excess output above this in Exp. 3 then becomes 3·098 g. nitrogen: applying the same method to the sulphur we find that 0·1867 g. was stored. The S : N ratio of this stored material is 1 : 16·5, which appears more reasonable than the ratio based on the first calculation. If we take 2·9 g. as the basal figure, a storage of 2·562 g. nitrogen and 0·2960 g. sulphur is found. The S : N ratio in this case is 1 : 9·6. This would bear out the hypothesis that the body stores sulphur first.

These three methods of calculation have each yielded a different S : N ratio, namely, 1 : 31·7, 1 : 16·5 and 1 : 9·6. It is most likely that the value 1 : 16·5 is the correct one but it is possible that the long period of nitrogen starvation may have so altered conditions that a sulphur-rich or a sulphur-poor protein has been stored. From the last two experiments it cannot be definitely

stated whether the body stores protein whose composition corresponds to that of the food on the one hand or to that of muscle on the other. On the whole the evidence from Exp. 2 with gelatin favours the view that the protein stored corresponds in its sulphur content to muscle tissue. The same can be said of Exp. 3 if we accept the basis of calculation which gives a S : N ratio of 1 : 16·5. There is a certain amount of evidence that in the process of storing the sulphur may be retained first and the nitrogen later, and thus, although the end result is to have a protein of average S : N ratio, for a short time during storage a sulphur-rich protein may exist; and *vice versâ* in the breakdown of protein.

Table II.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
23 Feb.	Basal	5·460	0·3472	280	1·491	0·3737	1 : 14·6	1 : 3·6
24 ,,	,, ...	5·544	0·3640	322	1·526	0·3242	1 : 17·1	1 : 3·6
25 ,,	,, ...	5·768	0·4480	302	1·562	0·4396	1 : 11·9	1 : 3·6
26 ,,	,, ...	5·537	0·4256	370	1·278	0·4836	1 : 11·1	1 : 4·0
27 ,,	,, ...	6·552	0·3752	467	1·278	0·5056	1 : 12·9	1 : 5·1
28 ,,	Gel. supsd (Exp. 4)	9·240	0·6992	457	1·313	0·7969	1 : 11·5	1 : 7·03
29 ,,	Basal	6·860	0·5992	312	1·171	0·5605	1 : 12·2	1 : 4·0
1 March	,,	6·468	0·6608	322	1·242	0·4461	1 : 14·4	1 : 5·2
2 ,,	,,	6·244	0·6104	462	1·207	0·4561	1 : 13·6	1 : 5·1
3 ,,	,,	6·076	0·5040	407	1·936	0·4561	1 : 13·6	1 : 5·8
4 ,,	Alb. supsd (Exp. 5)	7·252	0·4984	462	1·312	0·7912	1 : 9·1	1 : 5·5
5 ,,	Basal (,,)	8·344	0·5432	565	1·312	0·7473	1 : 11·1	1 : 6·3
6 ,,	,,	6·076	0·4816	360	1·278	0·4467	1 : 13·6	1 : 4·7
7 ,,	,,	6·272	0·4312	542	1·3845	0·4506	1 : 13·9	1 : 4·5

Exps. 4 and 5 were carried out with the same two proteins, superimposed in this case on a diet containing about 6·4 g. nitrogen, but without subsequent starvation. In Exp. 4, where the nitrogen output on the pre-days is unfortunately rather irregular, 70 g. gelatin containing 9·939 g. nitrogen and 0·3750 g. sulphur with a S : N ratio of 1 : 26·5 were superimposed. The S : N ratio was 1 : 12·9 on the day before superimposition (Table II). This ratio rose on the day gelatin was taken, which is in contrast with Exp. 2. The ratio rose to 1 : 11·5. The nitrogen and sulphur outputs both fell to their basal value by the fourth day, whilst the S : N ratio fell a little below its basal value. It will be noted that the S : N ratio was highest on the day of superimposition and lowest on the third day, so that there was clearly a nitrogen lag. The output of both nitrogen and sulphur was highest on the day of superimposition. The balance is calculated by subtracting the total nitrogen output on this diet from the output on the day of superimposition and subsequent days.

Nitrogen excreted in excess of basal = 2·996 g.
,, ingested = 9·939 ,,
,, retained = 9·939 − 2·996 = 6·943 ,,
Sulphur excreted in excess of basal = 0·3459 ,,
,, ingested = 0·3750 ,,
Sulphur retained = 0·3750 − 0·3459 = 0·0291 ,,
S : N ratio of stored material = 1 : 22·8.

In this case the material stored from gelatin is much poorer in sulphur than in Exp. 2. The conditions however are not quite identical. In Exp. 2 the body had lost protein and was in a relatively sulphur-poor condition, as is seen from the high S : N ratio before the gelatin was superimposed. In Exp. 4, on the other hand, the body is in nitrogen equilibrium with a protein of high sulphur content as is seen from the high S : N ratio on the day before superimposition (1 : 12·9). It is possible that the body was relatively saturated with sulphur groups and retained the nitrogen of the gelatin in order to lower the S : N ratio of the circulating protein to an average.

It will be noted that 6·9 g. of the 9·9 g. nitrogen of the gelatin ingested was retained. This is a high value for gelatin, which is considered to be a protein of low biological value, so that in all probability the basal diet improved the quality of the gelatin as a protein and made it suitable for storage.

Exp. 5 was carried out in the same way with egg white, 500 g. of this containing 19·296 g. nitrogen and 1·059 g. sulphur, with a S : N ratio of 1 : 9·6, being superimposed. On the day of superimposition the nitrogen output rose only 1·2 g., whilst the sulphur output did not exceed that on the day gelatin was taken; nevertheless the S : N ratio rose to 1 : 9·1. On the next day the nitrogen output rose 2·3 g. above the basal value, whilst the sulphur output and the S : N ratio fell a little. The maximum output of nitrogen is on the second day in contrast to what was seen in Exp. 4. This late rise seems to prove the extreme lability of the material stored from egg white. This also probably accounts for the rise in nitrogen output in Exp. 3 on the first day of starvation, instead of the fall seen in Exps. 1 and 2. This lability is possibly conditioned partly by the high sulphur content and partly perhaps by the nature of the sulphur moiety of egg white. The material stored and broken down again is evidently sulphur-rich, as the S : N ratio is high for the first two days, whilst on the third day, when the nitrogen output has reached its basal figure, the S : N ratio is 1 : 13·6, a little below the average for the experiment. It will also be noted that the sulphur has preceded the nitrogen output. The following figures show the balance.

Nitrogen excreted above the basal		= 3·440	g.
,, ingested		= 10·206	,,
,, retained = 10·206 − 3·440		= 6·766	,,
Sulphur excreted above the basal		= 0·6262	,,
,, ingested		= 1·059	,,
,, retained = 1·059 − 0·6262		= 0·4332	,,
S : N ratio of the stored material		= 1 : 15·2.	

In this experiment there has been a much better utilisation of sulphur than in Exp. 2. Again the body has selectively retained a material corresponding in its nitrogen and sulphur content to muscle tissue. It is necessary to remark that, although 0·4332 g. sulphur was retained, it does not follow that it was the sulphur of the egg white. It is quite possible that most of

the egg white sulphur was excreted and the sulphur of the basal diet stored in order to build up a protein with the nitrogen of the egg white. A later experiment lends support to this view.

Table III.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
22 March	Basal	11·116	0·504	495	1·948	0·610	1 : 18·2	1 : 5·7
23 „	„	10·500	0·504	440	1·5713	0·560	1 : 18·7	1 : 6·6
24 „	„	10·416	0·5152	482	1·929	0·599	1 : 17·3	1 : 5·3
25 „	„	11·284	0·470	462	2·214	0·588	1 : 19·1	1 : 5·0
26 „	Gel. supsd (Exp. 6)	16·520	0·739	607	2·692	0·901	1 : 18·3	1 : 6·1
27 „	Basal	14·448	0·750	600	2·258	0·626	1 : 23·0	1 : 6·3
28 „	„	13·272	0·694	565	2·498	0·670	1 : 19·7	1 : 5·3
29 „	„	11·200	0·560	487	2·857	0·615	1 : 18·1	1 : 3·9
30 „	„	11·680	0·539	467	2·333	0·582	1 : 20·0	1 : 5·0
31 „	Alb. supsd (Exp. 7)	13·888	0·649	590	2·491	1·198	1 : 11·6	1 : 5·5
1 April	Basal	15·232	0·772	565	2·737	1·187	1 : 12·8	1 : 5·7
2 „	„	11·312	0·694	402	1·944	0·7364	1 : 15·3	1 : 5·7
3 „	„	11·704	0·660	467	1·884	0·747	1 : 15·6	1 : 6·7

Exps. 6 and 7 (Table III) were the same as the preceding, but the basal diet was much richer in nitrogen. In Exp. 6 gelatin was taken in the same quantity as in Exp. 4. The average S : N ratio of the pre-days was 1 : 18·3, but the actual value on the day before superimposition was 1 : 19·1. On the day of superimposition it rose to its average value of 1 : 18·3. On the next day it fell to 1 : 23 and had returned to its average value two days later. It will be noted that there was a distinct nitrogen lag. The maximum output of nitrogen occurred on the day of superimposition and four days elapsed before it fell to the basal value. Five days elapsed before the sulphur came down to its basal value. The following is the balance for the period.

Nitrogen excreted above the basal value = 10·574 g.
„ ingested = 9·939 „
„ lost = 10·574 − 9·939 = 0·635 „
Sulphur excreted above the basal value = 0·4605 „
„ ingested = 0·3750 „
„ lost = 0·4605 − 0·3750 = 0·0850 „
S : N ratio of material lost = 1 : 10·2.

This small loss of both nitrogen and sulphur may either be real and due to the stimulating effect of gelatin on protein metabolism such as was observed by Cathcart and Green [1913], or it may simply fall within the limits of experimental error. The most important point is that when gelatin is superimposed on a basal diet of high nitrogen content, it is apparently completely rejected. In Exp. 7, 500 g. egg white containing 9·8 g. nitrogen and 1·204 g. sulphur with a S : N ratio of 1 : 11·6 were superimposed. On the day of superimposition the output of sulphur was nearly doubled, whilst that of the nitrogen rose only 2·4 g. The S : N ratio correspondingly rose to 1 : 11·6, almost double that of the pre-day (1 : 20). On the next day there is a repetition of what was seen in Exp. 5, the sulphur output falling a little whilst the

nitrogen output rose nearly 4 g. The same sharp fall is seen on the subsequent day but the sulphur never returned to its basal value. The S : N ratio continued to fall but failed to reach the original value. Taking as the basal value an average of the pre-days as in Exp. 7, there is the following balance:

Nitrogen excreted above the basal value	= 7·0 g.
„ ingested	= 9·8 „
„ stored = 9·8 − 7·0	= 2·8 „
Sulphur excreted above the basal value	= 1·5171 g.
„ ingested	= 1·2040 „
„ lost = 1·5171 − 1·2040	= 0·3131 „

In this case there is a considerable loss in sulphur with a small retention of nitrogen. It will be noted that the best retention of egg white both as regards the nitrogen and the sulphur was in Exp. 5, where the basal diet contained about 6 g. nitrogen. Possibly at the high level of protein intake in this experiment the body is at its maximum capacity for retaining nitrogen. In Exps. 4 and 5 the total nitrogen of the faeces rose in each case (about 1 g. above the average) on the day after superimposition. In Exps. 6 and 7, where the daily average output in the faeces was 2·236 g., the rise was rather more than 1 g. This increase in the nitrogen of the faeces may be due to non-absorption, in which case there would probably have been a lack of absorption of a corresponding quantity of sulphur. This would reduce the calculated quantity of material stored, but its quality would remain as found.

Table IV.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
14 April	Basal	4·312	0·408	327	2·101	0·3902	1 : 10·9	1 : 2·0
15 „	„	4·256	0·3584	412	1·505	0·2528	1 : 16·8	1 : 2·8
16 „	„	3·276	0·358	360	1·177	0·1978	1 : 16·5	1 : 2·7
17 „	„	3·024	0·364	350	1·079	0·2088	1 : 14·4	1 : 2·8
18 „	„	2·688	0·336	336	1·079	0·2088	1 : 12·8	1 : 2·4
19 „	Gel. supsd (Exp. 8)	4·564	0·476	345	0·568	0·4506	1 : 10·1	1 : 8·0
20 „	Basal	4·340	0·442	370	0·837	0·2528	1 : 17·1	1 : 5·1
21 „	„	3·668	0·341	445	0·894	0·2528	1 : 14·4	1 : 4·1
22 „	„	3·192	0·285	370	0·695	0·2308	1 : 13·8	1 : 4·5
23 „	„	2·912	0·280	345	0·624	0·2253	1 : 12·9	1 : 4·6
24 „	Alb. supsd (Exp. 9)	3·696	0·330	472	0·781	0·4506	1 : 8·2	1 : 4·7
25 „	Basal	3·528	0·375	370	0·454	0·5001	1 : 7·0	1 : 7·7
26 „	„	3·836	0·383	332	0·695	0·3792	1 : 10·1	1 : 5·5
27 „	„	3·388	0·296	420	0·569	0·3627	1 : 9·3	1 : 5·6
28 „	„	3·276	0·263	375	0·582	0·3462	1 : 9·4	1 : 5·6

The last group of experiments (Exps. 8 and 9, Table IV) were carried out with the nitrogen-free basal diet employed in the first group of experiments. The experiments were exactly similar to the earlier ones, except that there was no subsequent starvation. In Exp. 8, 70 g. gelatin, containing 9·939 g. nitrogen and 0·3705 g. sulphur, with a S : N ratio of 1 : 26·5, were superimposed when the nitrogen output was below 3 g. It will be noted that there was the same tendency for the S : N ratio to rise on the nitrogen-free diet.

On the day of superimposition the ratio rose to 1 : 10·1, the reverse of what was noted in Exp. 2. The possible reason of this is that in Exp. 2 the body had been on a nitrogen-free diet for seven days and, in addition, had starved two days. Under such conditions the tissues would store protein more readily. The following balance was found:

Nitrogen excreted above the basal value	= 5·236 g.
„ ingested	= 9·939 „
„ stored = 9·939 − 5·236	= 4·703 „
Sulphur excreted above the basal value	= 0·3683 „
„ ingested	= 0·3705 „
„ stored = 0·3705 − 0·3683	= 0·0022 „

In this case the nitrogen only has been stored. It is interesting to compare this experiment with Exp. 4, where the same quantity of gelatin was superimposed on a basal diet containing 6 g. nitrogen. In Exp. 4, 6·643 g. nitrogen and 0·0291 g. sulphur were stored. *A priori* the body should have retained more of the gelatin on a nitrogen-free diet than on a nitrogen-containing one. The reason for the poorer retention possibly lies in the fact that the basal diet in Exp. 4 may have improved the quality of the gelatin. It is also to be noted that even on the fifth day after superimposition the nitrogen had not quite returned to its basal value. This seems to show that the tissues were in need of nitrogen and were trying to utilise to the utmost an imperfect protein. The early excretion of sulphur with nitrogen lag is again seen in this experiment. In Exp. 9 egg white, containing 9·9619 g. nitrogen and 0·9995 g. sulphur with a S : N ratio of 1 : 9·9, were superimposed. Taking 2·912 g. nitrogen as the basal output we get:

Nitrogen excreted above the basal value	= 1·0218 g.
„ ingested	= 9·9619 „
„ stored = 9·9619 − 1·0218	= 8·9401 „
Sulphur excreted above the basal value	= 0·9123 „
„ ingested	= 0·9995 „
„ stored = 0·9995 − 0·9123	= 0·0872 „

S : N ratio of stored material = 1 : 101.

Again there is a poor retention of sulphur. The sulphur retention to cover starvation output is much greater than this in Exp. 3. The best retention of the sulphur of egg white was in Exp. 5, where 0·4322 g. was stored. In Exp. 5, however, it is likely that it was really the sulphur of the basal diet that was retained in order to build up some protein with the nitrogen of the egg white. In such a case the sulphur of the white may have been almost completely eliminated. The figures in Exp. 5 show a retention of 0·4322 g. sulphur when the white was superimposed, but clearly it does not necessarily follow that it was actually the sulphur of the white which was retained.

In fact from Exp. 9 it would seem as if the real value of the sulphur of egg white was low. Exp. 9 is a stringent experiment as the tissues were in great need of protein as compared with Exp. 5. In Exp. 9 the retention of nitrogen, however, was nearly 90 % of the intake.

This nitrogen was held firmly as the nitrogen output had not reached its basal value by the fifth day after superimposition, when the experiment was stopped as the diet became rather trying to take. The extreme difference in the quality of the material stored will be noted in Exps. 5 and 9. In Exp. 5 the material stored had a S : N ratio of 1 : 15·2, corresponding to the average for muscle tissue, whilst in Exp. 9 the retained material consisted of nitrogen alone (S : N = 1 : 101). It will be noted that the highest output of sulphur is on the day after superimposition, whilst in the case of the nitrogen it is on the second day after taking the egg white. The same lability of the material stored is seen as in Exps. 5 and 7, but, owing to the poverty in nitrogen of the body the material is retained a day longer than in the two previous experiments. This experiment also shows clearly the delay in the nitrogen excretion after that of sulphur. The sulphur output never reached its basal value although it had been eliminated almost completely.

The results of these last two experiments taken in conjunction with Exps. 4 and 5 show the effect of a basal diet in improving the quality of such proteins as gelatin and egg white in such a way as to render them more suitable for storage.

Throughout all these experiments the output of uric acid was followed. In general it will be noted that the lowest output of uric acid was on the nitrogen-free diet, and the highest on the high-nitrogen diet. The total variation in the uric acid output is nearly 300 %, the lowest output being 205 mg. on the first day of starvation, and the highest, 600 mg. on the day gelatin was taken in Exp. 6. The output in general varies with the level of nitrogen equilibrium at the time. This indicates that one of the factors governing the output of uric acid must be the metabolism not only of the ingested protein but also of the ingested carbohydrate and fat. On returning to the nitrogen-free diet after starving a rise in uric acid output is seen. According to Cathcart the presence of carbohydrate is supposed to aid in the synthesis of uric acid or its precursor from some nitrogenous source, possibly arginine and histidine [Ackroyd and Hopkins, 1916]. Folin classed uric acid as the product of a more or less constant metabolic process which was little influenced by food ingestion. This position can possibly be held for creatinine which appears to be constant in output, whether on a high- or low-nitrogen diet. The function associated with creatinine formation is probably one which must go on at all costs and within narrow limits. The output of uric acid, however, appears to be intimately associated with the metabolism of the three energy-yielding foodstuffs.

Conclusions.

The first question to be answered was whether there was a delay in the excretion of ingested protein, such as might lead to the conclusion that it was stored. This delay has been observed in the case both of gelatin and egg white. The ease with which these materials are stored and the duration of their retention in the body varies, however, with the nutritive condition at the time. In Exp. 4, 60 % of the gelatin was stored and the remaining 40 % was all eliminated in three days. This was with nitrogen equilibrium in the neighbourhood of 6 g. *per diem*. In Exp. 6, with nitrogen equilibrium at 11 g. *per diem*, the whole of the gelatin was eliminated in three days. In Exp. 8, where the body was on a nitrogen-free diet and when it was in the poorest nutritive condition as regards nitrogen of all three experiments, the amount stored was less than 50 %. It will be noted that it took at least five days for the rejected part to be eliminated. The possible explanation of this variation may be that in Exp. 6 the body was flushed to its maximum capacity with protein and was incapable of storing any more. In Exp. 4 the nitrogen condition of the body was below its optimum so that gelatin was stored and in addition its quality was improved by the nitrogen of the basal diet. In Exp. 8 the tissues, being poor in nitrogen, retained as much of it as possible. The amount stored was less than in Exp. 4 with a nitrogen-containing diet, because there was no protein in the basal diet to improve its quality for storage.

As regards the egg white it was best retained in Exp. 9, where the body was in need of protein, 90 % of the nitrogen intake being retained. The storage of sulphur, however, is different. In Exp. 9 the S : N ratio of the stored material is 1 : 101, practically no sulphur being retained. In Exp. 5 the S : N ratio of the stored material is 1 : 15·1, 40 % of the sulphur being retained. It was probably the sulphur of the basal diet which was mostly retained. The main points to be noted are:

1. The nitrogen of egg white can be stored independently of the sulphur, hence it follows that a sulphur-poor material may be retained in the tissues.

2. If the protein superimposed contain sulphur of proper quality much sulphur may be retained along with the nitrogen ingested to form a material whose S : N ratio is similar to that of tissue (muscle) protein.

It would seem to be clear from these experiments that the changes which occur in the protein molecule affect first the sulphur fraction and then the nitrogen. In the process of storage the sulphur is retained first and the nitrogen later. In breakdown the reverse occurs. If an attempt is made to visualise the possible changes which occur one cannot consider the sulphur-containing group as a nucleus around which sulphur-free nitrogen-containing bodies are built because in the catabolic phase one would require to assume that the sulphur moiety of the protein molecule would be attacked last and all the evidence is against such a view. Rather must the molecule be looked

on as a chain of amino acids with the sulphur-containing group interposed at certain links and conferring lability on the molecule.

It has been argued that the early excretion of sulphur is due to the fact that the breaking off of the sulphur molecule precedes the deamination of the amino group. Wolf and Osterberg [1911], however, found that when cystine was administered to an animal the nitrogen was eliminated before the sulphur. The natural inference from this experiment is that the sulphur was protected until the amino group had been removed. Another fact, which appears to support the view that there is no causal relationship between the lag in the output of nitrogen as compared with sulphur and the process of deamination and oxidation, is that a similar discrepancy in output occurs when anabolic phenomena predominate, as in the storage or retention of protein within the body. In this storage phenomenon it must be noted, however, that the primary preference in retention is given to the sulphur part of the molecule. In view of the fact that this lag in the nitrogen is associated both with the anabolism of food protein and the catabolism of protein (tissue protein or food protein built up into some complex), but not with the catabolism of food protein in the amino acid form, it would seem to follow that, when a differential delay in excretion is observed after the ingestion of food protein, it may be assumed that this food protein must have been built up into some form of protein in the tissues and not immediately metabolised as amino acids.

In this connection it must be born in mind that the nitrogen and sulphur ingested in the form of protein are not excreted forthwith, but may be retained in the body for three to five days. Hence it must follow that the retention takes place in some protected form and the normal assumption is that it has been taken into the cells and retained there either to become an integral part of the cell content or simply to be added on to form a kind of prosthetic group. The main point is that it cannot be regarded as remaining and circulating in the tissue fluids in a highly labile form.

As regards the sparing effect of gelatin on the nitrogen output in starvation it was found that 58 % of the excess output due to starvation could be spared by gelatin. This result is much superior to those previously given and may be due simply to the methods of calculation adopted in the present series, although it is believed that the present value is nearer the truth than the older ones.

Summary.

1. There is a delay in the excretion of ingested protein extending over several days. This occurs whether the basal diet contains nitrogen or not and even when the feeding is followed by a short period of starvation.

2. The sulphur moiety of the protein molecule is the first to be mobilised both in the storage and breakdown of protein. There is always a delay in the excretion of nitrogen as compared with sulphur. The delay in the excretion of sulphur, which is sometimes found, is not due to a delay in the utilisation of

the sulphur moiety of the protein. It is due to an active preferential retention of sulphur in the tissues.

3. There is a certain amount of evidence that the retained material can exercise a sparing action on protein breakdown in the first few days of starvation.

4. Ingested protein may be retained in the body in some relatively complex form.

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L. A NOTE ON THE KINETICS OF ENZYME ACTION.

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The equation of Michaelis and Menten [1913] has been applied with success by Kuhn [1924] and others to numerous cases of enzyme action. It is therefore desirable to examine its theoretical basis. Consider the irreversible reaction $A \rightarrow B$, unimolecular as regards A, and catalysed by an enzyme. Suppose one molecule of A to combine reversibly with one of enzyme, the compound then changing irreversibly into free enzyme and B, where B may represent several molecules. We may represent this as:

$$\begin{array}{ccccc} A + E & & \rightleftharpoons & AE & \rightarrow B + E. \\ (a-x)\,(e-p) & & & p & x \end{array}$$

Now let a be the initial concentration of A, e the total concentration of enzyme, x the concentration of B produced after time t, and p the concentration of enzyme combined with substrate at time t. We suppose e and p to be negligibly small compared with a and x. Then by the laws of mass action

$$\frac{dp}{dt} = k_1 (a-x)\,(e-p) - k_2 p - k_3 p,$$

where k_1, k_2, k_3 are the velocity constants of the reactions

$$A + E \rightarrow AE, \quad AE \rightarrow A + E, \quad \text{and} \quad AE \rightarrow B + E,$$

respectively. Now since p is always negligible compared with x and $a - x$, its rate of change must, except during the first instant of the reaction, be negligible compared with theirs.

For during the remainder of the reaction p diminishes from a value not exceeding e to zero, whilst x increases from zero to a. Thus the average value of $-\frac{dp}{dt} \div \frac{dx}{dt}$ is less than $\frac{e}{a}$. And provided $\frac{e}{a}$ is small it is clear that if the amount of combined enzyme decreased for a measurable time at a rate comparable with that of its substrate the reaction would come to an end. To take a concrete example Kuhn [1924] calculates that a yeast saccharase molecule at 15° and p_H 4·6 can invert 100 or more molecules of sucrose per second. Even if the enzyme concentration is so unusually large that the inversion of a strong sucrose solution is half completed in 10 minutes, $\frac{a}{e}$ cannot be less than 120,000, and if $-\frac{dp}{dt}$ attained 1 % of the value of $\frac{dx}{dt}$ for 1 second the

reaction would stop owing to all the enzyme being set free. (Actually it may be shown that

$$\frac{dp}{dt} = \frac{-k_2(k_2+k_3)\,e\,(a-x)}{k_1\left(a-x+\frac{k_2+k_3}{k_1}\right)^2}.)$$

Hence $$k_1(a-x)(e-p) - k_2p - k_3p = 0.$$

$$\therefore\ p = \frac{k_1 e\,(a-x)}{k_2+k_3+k_1(a-x)}$$

$$= \frac{e\,(a-x)}{a-x+\frac{k_2+k_3}{k_1}}$$

$$\therefore\ \frac{dx}{dt} = k_3p = \frac{k_3 e\,(a-x)}{a-x+\frac{k_2+k_3}{k_1}}.$$

This is Michaelis and Menten's [1913] equation, $(k_2 + k_3)/k_1$ representing their constant K_s. They assume that the reaction

$$A + E \rightleftharpoons AE$$

is always practically in equilibrium, and K_s its equilibrium constant, *i.e.* that k_3 is negligible in comparison with k_2. Van Slyke and Cullen [1914] on the other hand assumed that the first stage of the reaction was irreversible, *i.e.* $k_2 = 0$, and arrived at the same equation. It is clear, however, that data as to the course of a reaction can give no indication of the ratio of k_2 and k_3, though when the velocity of the observed reaction, and hence k_3, is very large, the upper limit to k_2 deducible from the kinetic theory may possibly prove to be of the same order of magnitude.

It may be remarked that with this modification of their theory, Michaelis and Menten's analysis of the effects of the products of the reaction, or other substances which combine with the enzyme, still holds good.

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